Regulation of prostate cancer cell function by activators of AMP-activated protein kinase

Thesis submitted by

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Dicit ei Iesus: Ego sum via, et veritas, et vita. Nemo venit ad Patrem, nisi per me.

Ioannes 14:6, VULG

Ego enim scio cogitationes quas ego cogito super vos, ait Dominus, cogitationes pacis et non afflictionis, ut dem vobis finem et patientiam.

Ieremiah 29:11, VULG

Jesus answered, "I am the way and the truth and the life. No one comes to the Father except through me.

John 14:6, NIV

For I know the plans I have for you," declares the LORD, "plans to prosper you and not to harm you, plans to give you hope and a future.

Jeremiah 29:11, NIV

To my family

Abstract

AMP-activated protein kinase (AMPK) is a key regulator of cell energy homeostasis. More recently, it has become apparent that AMPK regulates cell proliferation, migration and inflammation. Previous evidence has suggested that AMPK may influence proliferation and invasion by regulating the pro-proliferative mitogen-activated protein kinases (MAPKs). However, the mechanisms underlying this crosstalk between AMPK and MAPK signalling are not fully understood. As AMPK activation has been reported to have anti-proliferative effects, there has been increasing interest in AMPK activation as a therapeutic target for tumourigenesis.

The aim of this study was to investigate whether AMPK activation influenced prostate cancer (PC) cell line proliferation, migration and signalling. Therefore, different PC cell lines were incubated with two structurally-unrelated molecules that activate AMPK by different mechanisms, AICAR and A769662. Both chemicals activated AMPK in a concentration- and time-dependent manner in PC3, DU145 and LNCaP cell lines. AMPK activity as assessed by AMPK activating phosphorylation as well as phosphorylation of the AMPK substrate ACC increased along with tumour severity in PC biopsies. Furthermore, both activators of AMPK decreased cell proliferation and migration in the androgen-independent PC cell lines PC3 and DU145. Inhibition of proliferation by A769662 was attenuated in *AMPK α1*$^{-/-}$ *AMPK α2*$^{-/-}$ knockout (KO) mouse embryonic fibroblasts (MEFs) compared to wild type (WT) MEFs, and the inhibitory effect on migration of AICAR lost significance in PC3 cells infected with adenoviruses expressing a dominant negative AMPK α mutant, indicating these effects are partially mediated by AMPK. Furthermore, long-term activation of AMPK was associated with inhibition of both the phosphatidylinositol 3'-kinase/protein kinase B (PI3K/Akt) signalling pathway in addition to the extracellular signal-regulated kinase 1/2 (ERK1/2) signalling pathway. Indeed, the actions of AMPK activators on PC cell line viability were mimicked by selective inhibitors of Akt and ERK1/2 pathways.

In contrast to the effects of prolonged incubation with AMPK activators, short-term incubation with AMPK activators had no effect on epidermal growth factor (EGF)-stimulated ERK1/2 phosphorylation in PC cell lines. In addition, AMPK activation did not influence phosphorylation of the other MAPK family members p38 and JNK. Interestingly, both AICAR and A769662 decreased EGF-stimulated ERK5 phosphorylation in PC3, DU145 and LNCaP cells as assessed with an anti-phospho-ERK5 antibody. Further characterisation of this effect indicated that prior stimulation with the AMPK activators had no effect on ERK5 phosphorylation stimulated by transient transfection with a constitutively active ERK5 kinase (MEK5DD), which represents the only known canonical kinase for ERK5. Intriguingly, the pattern of EGF-stimulated ERK5 phosphorylation was distinct from that mediated by MEK5DD activation of ERK5. This finding indicates that AMPK activation inhibits EGF-stimulated ERK5 phosphorylation at a point at or above the level of MEK5, although why EGF and constitutively active MEK5 stimulate markedly different immunoreactive species recognised by the anti-phospho-ERK5 antibody requires further study. A769662 had a tendency to reduce EGF-stimulated ERK5 phosphorylation in WT MEFs, yet was without effect in MEFs lacking AMPK. These data indicate that AMPK may underlie the effect of A769662 to reduce EGF-stimulated ERK5 phosphorylation.

Prolonged stimulation of PC cell lines with AICAR or A769662 inhibited EGF-stimulated Akt Ser473 phosphorylation, whereas only incubation with A769662 rapidly inhibited Akt phosphorylation. This difference in the actions of the different AMPK activators may suggest an AMPK-independent effect of A769662. Furthermore, AICAR increased phosphorylation of Akt in WT MEFs, an effect that was absent in MEFs lacking AMPK, indicating that this effect of AICAR may be AMPK-dependent.

Taken together, the data presented in this study suggest that AMPK activators markedly inhibit proliferation and migration of PC cell lines, reduce EGF-stimulated ERK1/2 and Akt phosphorylation after prolonged incubation and rapidly inhibit ERK5 phosphorylation. Both AMPK activators exhibit a number of effects that are likely to be independent of AMPK in PC cell lines, although

inhibition of ERK1/2, ERK5 and Akt may underlie the effects of AMPK activators on proliferation, viability and migration. Further studies are required to understand the crosstalk between those signalling pathways and their underlying significance in PC progression.

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Declaration

I declare that I am the author of this thesis. The experiments were designed by me with advice from my supervisors Dr Ian P Salt and Prof Hing Y Leung.

All the laboratory work and data analysis were carried out by myself unless stated otherwise. Specifically the experimental data presented in Figures 3.1 (partly), 3.14, 4.3, 4.16 and 6.3 were generated by Dr Yashmin Choudhury. This thesis in its entirety is my own original work, which has not been submitted for any higher degree at the University of Glasgow or any other institution.

Zichu Yang

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Abbreviations

ACC	Acetyl-CoA Carboxylase
AICAR	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AIS	Auto Inhibitory Sequence
AMPK	AMP-activated Protein Kinase
ANOVA	Analysis of variance
AR	Androgen Receptor
ASK	Apoptosis Signal-regulating Kinase
ATF	Activating Transcription Factor
BAD	Bcl-2-associated Death Promoter
BrdU	Bromodeoxyuridine
CaMKK	Ca^{2+}/calmodulin-dependent Protein Kinase Kinase
CBS	Cystathionine Beta Synthase
CRPC	Castration-resistant Prostate Cancer
DHT	Dihydrotestosterone
DN	Dominant Negative
EC_{50}	Half Maximal Effective Concentration
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular-signal-regulated Kinase
ET	Endothelin
ETS	E26 Transforming Sequence
GBD	Glycogen Binding Domain
HMGR	Hydroxymethylglutaryl-CoA Reductase
HSL	Hormone-sensitive Lipase
HSP	Heat-shock Proteins
IFU	Infection Forming Unit
IGF	Insulin-like Growth Factor
IR	Ionizing Radiation
JNK	c-Jun N-terminal Kinase
KO	Knockout
LKB1	Liver Kinase B1

MAPK	Mitogen-activated Protein Kinase
MAPKAPK	Mitogen-activated Protein Kinase Activated Protein Kinase
MAPKK	Mitogen-activated Protein Kinase Kinase
MAPKKK	Mitogen-activated Protein Kinase Kinase Kinase
MEF	Mouse Embryonic Fibroblast
MEF2C	Myocyte Enhancer Factor 2C
MEK	Mitogen-activated Protein Kinase Kinase
MEKK	Mitogen-activated Protein Kinase Kinase Kinase
MLK	Mixed-lineage Kinase
mTOR	Mammalian Target of Rapamycin
mTORC	Mammalian Target of Rapamycin Complex
PC	Prostate Cancer
PDK	3-Phosphoinositide-dependent Protein Kinase
PI3K	Phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKA	Protein Kinase A
PSA	Prostate Specific Antigen
PTEN	Phosphatase and Tensin Homologue
Rheb	Ras homolog enriched in brain
RSK	Ribosomal S6 Kinase
RTK	Receptor Tyrosine Kinase
SBS	Subunit Interacting Sequence
SGK	Serine/threonine-protein Kinase
SID	Subunit Interacting Domain
siRNA	Small Interfering RNA
TAK	TGF-β Activated Kinase
TGF	Transforming Growth Factor
TMA	Tissue Microarray
TORC	Target of Rapamycin Complex
TPRSS2	Transmembrane Protease Serine 2
TSC	Tuberous Sclerosis Protein
WT	Wild Type
ZMP	5-aminoimidazole-4-carboxamide Ribonucleotide

Chapter 1. Introduction

1.1 Prostate cancer

1.1.1 Epidemiology

Prostate cancer (PC) is the most common non-skin cancer in men worldwide, with an approximate incidence of 200, 000 per year (Parkin *et al.*, 2001, Hsing and Chokkalingam, 2006, Chamie *et al.*, 2015). In the UK, the prevalence is around 50 cases per 100,000 population (Stewart and Finney, 2012), with rising incidence, making PC the most common malignancy in males (Cancer Research UK, 2013). In the US, the age-adjusted incidence is even higher, with more than 150 per 100, 000 men per year (Siegel *et al.*, 2015). PC is also the second most common cause of cancer death in men (Stewart and Finney, 2012). It is usually common in males aged over 50 years (Epstein and Lotan, 2015), and the incidence varies widely among ethnic groups and countries (Gronberg, 2003), which is thought to result from a complex interaction between genetic and environmental factors. However, only a handful of PC risk factors are known, which include age, African ethnicity, family history, inflammation and infection (De Marzo *et al.*, 1999, Gronberg, 2003, Zeegers *et al.*, 2003, Hsing and Chokkalingam, 2006, Jani *et al.*, 2008, Klein and Silverman, 2008, Stephenson and Klein, 2016). Using molecular epidemiological approaches, many biomarkers have been identified as being linked to increased PC risk, including androgen receptor (AR), oestrogen, insulin-like growth factor (IGF), leptin and Vitamin D (Chung and Leibel, 2006, Ribeiro *et al.*, 2006, Nelles *et al.*, 2011, Uzoh *et al.*, 2011, Schwartz, 2013, Sun and Lee, 2013, Stephenson and Klein, 2016).

1.1.2 Pathology

The prostate is a globular fibromuscular gland constituted of multilayered connective tissue (Myers, 2001, Ginzburg *et al.*, 2016). Adenocarcinoma is the most common malignancy in the prostate, which accounts for about 95% (Hamdy and Robson, 2010). The most common genetic deficits are either mutation or deletion leading to activation of the phosphatidylinositide 3-kinase (PI3K)/Akt signalling pathway and the fusion of transmembrane protease serine 2 (*TPRSS2*)

and E26 transforming sequence (*ETS*) gene (Clark and Cooper, 2009, Tomlins *et al.*, 2009, Epstein and Lotan, 2015). AR is a key regulator of PC in terms of cancer development and progression in both hormone-sensitive and castration-resistant PC (CRPC) (Heinlein and Chang, 2004). PCs are all androgen-dependent for tumour growth and survival initially, although some of them become androgen-independent eventually (Feldman and Feldman, 2001). Recent studies suggest AR promotes PC by up-regulation of metabolism, biosynthesis and cell cycle regulators (Massie *et al.*, 2011).

Prostatic intraepithelial neoplasia (PIN) consists of normally structured prostate acini ducts lined by cytologically atypical cells (Ross and Rodriguez, 2016). PIN can be classified into high-grade PIN and low-grade PIN, and is considered as a pre-adenocarcinoma lesion of the prostate (McNeal and Bostwick, 1986, McNeal, 1989, Ross and Rodriguez, 2016).

The most commonly used clinical staging classification of PC is the TNM (tumour, node, metastasis) system which assesses three components, including the extent of primary tumour, status of regional lymph nodes and distant metastases (Epstein and Lotan, 2015, Scher and Eastham, 2015, Loeb and Eastham, 2016) (Table 1.1). The Gleason score system is based on the pattern of the glandular structure within the prostate as identified at low magnification (Mellinger *et al.*, 1967, Gleason and Mellinger, 1974). The modified Gleason scoring system identifies the architectural patterns from grade 1 to 5, with 5 being the most undifferentiated tumour (Epstein *et al.*, 2005). The most common and highest-grade patterns were then added to give a total Gleason score (Epstein *et al.*, 2005). Grouping of Gleason scores based on prognoses has been used in recent practice, with Grade Group I being the most favourable and Grade Group V being the least favourable (Pierorazio *et al.*, 2013). The TNM classification system is supplemented by the prostate specific antigen (PSA) level and Gleason score to classify newly diagnosed cases into prognostic groups (Loeb and Eastham, 2016).

TNM designation	Anatomic findings
Primary tumour (T)	
Tx	Cannot be assessed
T0	No evidence
T1	Clinically inapparent
T1a	Involvement ≤ 5% of resected tissue
T1b	Involvement > 5% of resected tissue
T1c	Identified by needle biopsy
T2	Confined within prostate
T2a	Half lobe or less involvement
T2b	More than half lobe involvement but unilateral
T2c	Both lobes involvement
T3	Extraprostatic extension
T3a	Extracapsular extension
T3b	Seminal vesicle invasion
T4	Adjacent structure invasion
Regional lymph nodes (N)	
Nx	Not assessed
N0	Not involved
N1	Metastases
Distant metastases (M)	
M0	Not present
M1	Present
M1a	Non regional lymph nodes
M1b	Bone
M1c	Other site

Table 1.1 The clinical TNM (Tumour, Node, Metastasis) staging system for prostate cancer

The clinical stage of prostate cancer is assessed using the TNM system. Adapted from (Edge *et al.*, 2010).

1.1.3 Current therapeutic approaches

There is no approved preventive measure for PC to date (Scher and Eastham, 2015). PSA is a glycoprotein widely used as a serum tumour marker in PC (Neal and Shaw, 2013, Dark and Abdul Razak, 2014). However, its reliability to detect early stage PC is limited and there remains controversy over the usefulness of PSA alone as a screening tool (Neal and Shaw, 2013). PSA screening in combination with digital rectal examination is a widely used clinical approach for diagnosis and risk assessment (Schmid *et al.*, 2004, Smith *et al.*, 2007, Heidenreich *et al.*, 2008). Other blood-based biomarkers that have been proposed for PC include free PSA and its isoforms, prostate specific membrane antigen, human kallikrein 2, endoglin and circulating tumour cells (Morgan *et al.*, 2016). In addition, urine-based biomarkers such as PC antigen 3 and annexin A3, as well as tissue-based biomarkers such as α-methylacyl coenzyme A racemase are also being developed and investigated (Morgan *et al.*, 2016). Different treatments for PC are offered to patients depending on their clinical situation such as age, Gleason score, tumour stage, PSA level and pathological status (Heidenreich *et al.*, 2008). Management strategies for localised PC include active surveillance or watchful waiting, radical prostatectomy, radiation therapy and focal therapy such as brachytherapy and ablation (Ahmed and Emberton, 2016, Carter and Dall'Era, 2016, Catalona and Han, 2016, D'Amico *et al.*, 2016). Adjuvant therapies including hormonal therapy are used for locally advanced PC and/or biochemically recurrent PC (Lee and Thrasher, 2016, Meng and Carroll, 2016). Androgen deprivation therapy is the hormonal therapy available currently functioning by reducing AR activity (Nelson, 2016). Although androgen ablation is beneficial in PC, the therapy itself also generated a new disease status, CRPC (Nelson, 2016). CRPC makes the management of the patient in this group more complicated; many therapeutic approaches are still under evaluation, these include cytotoxic chemotherapy, immunotherapy and targeted therapy (Antonarakis *et al.*, 2016).

1.1.4 Oncogenesis

PC is believed to originally initiate from prostate epithelial cells (Goldstein *et al.*, 2010), of which luminal cells are widely accepted as sites of origin (Parsons *et al.*, 2001). Studies also suggest that basal cells also have the potential to contribute to carcinogenesis (Goldstein *et al.*, 2010). The progression of oncogenesis can be characterised by gene mutation in the form of activating mutation of oncogenes or inactivation of tumour suppressing genes (Ahmad *et al.*, 2012). Many genes have been found to be mutated in PC including p53, phosphatase and tensin homologue (*PTEN*), retinoblastoma (*RB*), ras, p16 (*CDKN2A*), *CTNNB1* and *AR* (Isaacs and Kainu, 2001). Nevertheless, a single mutation alone is not sufficient for transformation and carcinogenesis (Ahmad *et al.*, 2012).

AR is a key regulator of PC in terms of cancer development and progression in both hormone-sensitive and CRPC (Heinlein and Chang, 2004). Although recent technology has advanced analysis of not only overall protein levels, but also post-translational modifications, it is still difficult to identify the mechanisms of oncogenesis progression (Endoh *et al.*, 2012). Prostate carcinogenesis is complex and involves multiple genes/pathways, yet the exact molecular basis of this complex interaction remains to be fully determined.

1.1.5 Aberrant signalling pathways involved in prostate cancer

A number of abnormalities involving distinct signalling pathways have been identified in PC *in vitro* and preclinical animal models, some of which are potential therapeutic targets (Ramsay and Leung, 2009). Examples of aberrant signalling cascades include AR, PI3K/Akt, mitogen-activated protein kinase (MAPK) pathways, Wnt pathway, endothelin (ET) axis, Src family kinase, heat-shock proteins (HSP) and anti-apoptotic proteins, often as consequences of inappropriate ErbB receptor, fibroblast growth factor receptor and IGF-1 signalling (Mehta *et al.*, 2001, Kinkade *et al.*, 2008, Ramsay and Leung, 2009, Ahmad *et al.*, 2011, Ramsay *et al.*, 2011, Takahashi *et al.*, 2011).

1.1.5.1 Role of androgens

Androgen, one of the sex hormones, is responsible for the normal function of prostate tissue, such as growth, development and homeostasis (Ross and Rodriguez, 2016). The predominant androgen found in the prostate gland is dihydrotestosterone (DHT) (Ross and Rodriguez, 2016). AR is a nuclear receptor, which can be activated by binding androgen hormones, such as testosterone and DHT (Lu *et al.*, 2006). Androgen binds to the AR in the cytoplasm after passive diffusion through the cell membrane. Ligand-bound AR is subjected to post-translational modifications, such as phosphorylation, dimerisation and subsequent active transport to the nucleus (Lonergan and Tindall, 2011, Ross and Rodriguez, 2016). AR in the nucleus then acts as a transcription factor, directly and/or indirectly binding to DNA, leading to changes in gene expression (Mills, 2014). The AR signalling pathway has been recognised as one of the most important pathways in prostate carcinogenesis (Newmark *et al.*, 1992, Gnanapragasam *et al.*, 2000, Heinlein and Chang, 2004, Lonergan and Tindall, 2011, Massie *et al.*, 2011).

1.1.5.2 Role of PI3K/Akt

The PI3K family is a large group of lipid kinases (Fruman *et al.*, 1998, Hennessy *et al.*, 2005), consisting of three classes, namely Class IA, IB, II and III (Hennessy *et al.*, 2005). The PI3Ks are heterodimers with a catalytic subunit and an adapter/regulatory subunit and can be activated by pathways stimulated by growth factors binding at receptor tyrosine kinases (RTKs) or by G-proteins (Vanhaesebroeck and Waterfield, 1999, Katso *et al.*, 2001). Class I PI3K family members catalyse the production of phosphatidylinositol-3,4,5-trisphosphate (PIP3) production from phosphatidylinositol-4,5-bisphosphate. PIP3 then activates the serine-threonine protein kinase Akt (Manning *et al.*, 2002, Osaki *et al.*, 2004, Hemmings and Restuccia, 2012). There are three distinct isoforms of Akt (Akt1, Akt2 and Akt3) (Staal *et al.*, 1988, Cheng *et al.*, 1992, Nakayama *et al.*, 2006). Each of the isoforms consists of a plekstrin homology (PH) domain at the N-terminus, a kinase domain and a regulatory domain at its C-terminus (Osaki *et al.*, 2004). PIP3 activates Akt by initiating conformational changes in Akt, leading

to the exposure of phosphorylation sites in both the kinase domain (Thr308 in Akt1) and the regulatory domain (Ser473 in Akt1) (Alessi *et al.*, 1996). The constitutively active phosphoinositide-dependent kinase (PDK) 1 phosphorylates Thr308 in the kinase domain to stabilise the activation loop whereas PDK2s phosphorylate the hydrophobic regulatory domain (Alessi *et al.*, 1996, Blume-Jensen and Hunter, 2001). This dual phosphorylation on both domains is essential for the full activation of Akt (Hennessy *et al.*, 2005). Several kinases have been proposed as PDK2s, including mammalian target of rapamycin complex (mTORC) 2, integrin-linked kinase (ILK) and protein kinase C (Lynch *et al.*, 1999, Kawakami *et al.*, 2004, Sarbassov *et al.*, 2005). Of these, mTORC2 has been demonstrated to be an *in vivo* phospho-Akt Ser473 kinase (Sarbassov et al., 2005). Akt activation directly inhibits the tuberous sclerosis protein (TSC) complex, leading to Ras homolog enriched in brain (Rheb) activation, which can then stimulate mTORC1 activity (Ouwens *et al.*, 1999, Sekulić *et al.*, 2000, McManus and Alessi, 2002, Dibble and Cantley, 2015) (Figure 1.1). The PI3K/Akt signalling pathway regulates several cellular events including cell growth, cell cycle regulation, apoptosis, metabolism, translation and proliferation (Vivanco and Sawyers, 2002, Luo *et al.*, 2003, Hennessy *et al.*, 2005). Over-activation of the PI3K/Akt pathway has been proposed to play a vital role in PC (Murillo *et al.*, 2001, Gao *et al.*, 2003, Shukla *et al.*, 2007, Sarker *et al.*, 2009, Carver *et al.*, 2011).

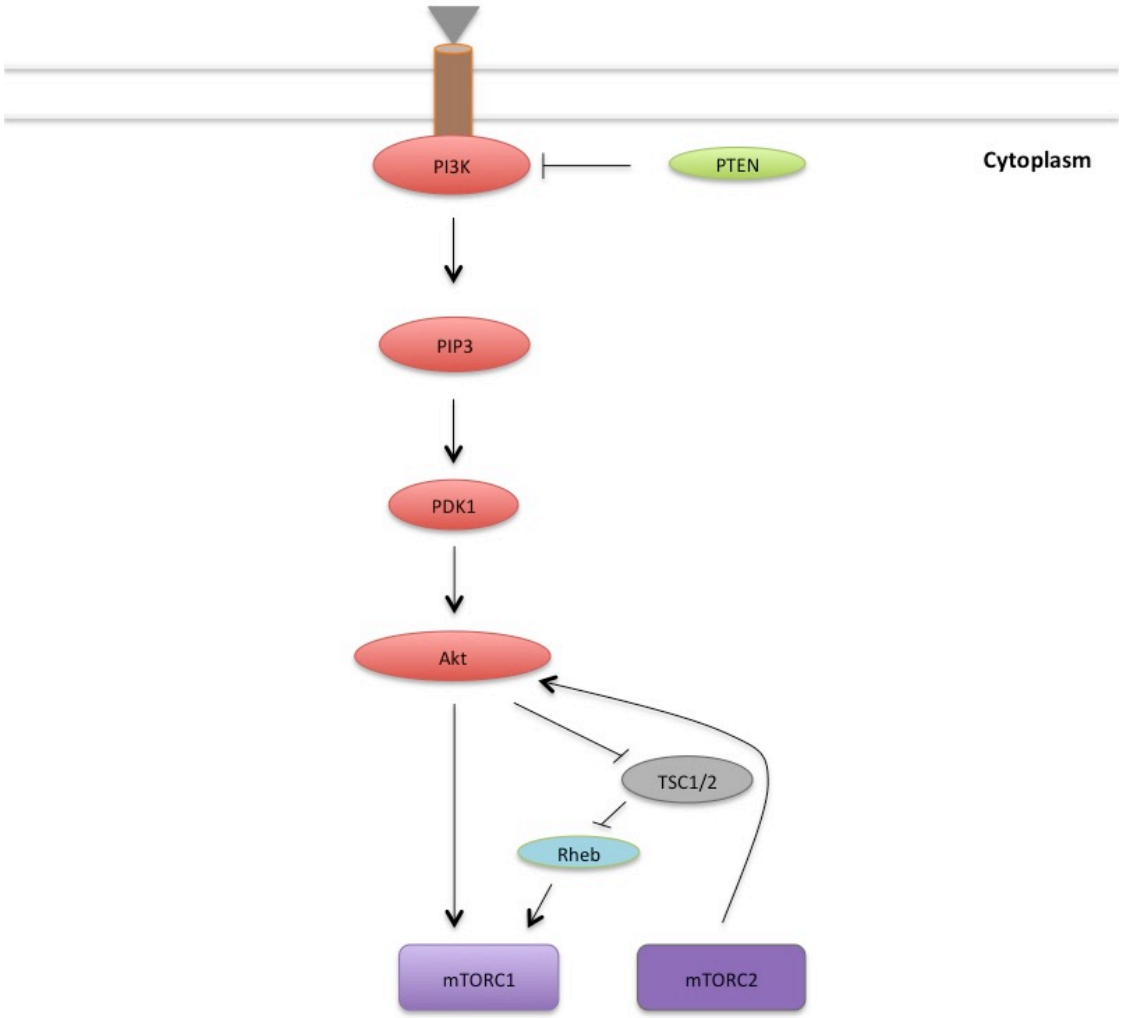


Figure 1.1 Simplified outline of the PI3K/Akt signalling pathways

After activation by growth factor or cytokine (grey triangle), PI3K synthesises PIP3. PIP3 then recruits PDK1 and Akt to the plasma membrane and stimulates their subsequent phosphorylation, leading to mTORC1 activation through both inhibition of TSC1/2 (grey) and the subsequent inhibition of Rheb (light blue) and direct phosphorylation and activation mTORC1. There is also a positive feedback loop where mTORC2 phosphorylates and activates Akt. The PI3K/Akt signalling cascade is shown in red. PTEN, a negative regulator of PI3K, is shown in green. Arrow-headed lines denote activation and bar-headed lines denote inhibition.

1.1.5.3 Role of mitogen-activated protein kinases

MAPKs belong to a family of serine/threonine protein kinases, responding to extracellular stimuli for the regulation of cellular functions such as proliferation, differentiation, migration and apoptosis (Pearson *et al.*, 2001, Turjanski *et al.*, 2007). MAPKs are involved in many diseases including chronic inflammation and multiple cancer types (Kyriakis and Avruch, 2001, Zarubin and Han, 2005). Consisting of a three-tier kinase module system, the separate signalling cascade for each group of MAPK involves the consecutive activation of a specialised MAPK kinase kinase (MAPKKK or MAP3K), and MAPK kinase (MAPKK, MAP2K or MEK), after stimulation by an extracellular stimulus (Pearson *et al.*, 2001, Dhillon *et al.*, 2007). MAPKK phosphorylates and activates the appropriate MAPK on a Thr-X-Tyr tripeptide motif, and activated MAPKs phosphorylate and regulate many cellular substrate proteins including nuclear transcription factors (Turjanski *et al.*, 2007) (Figure 1.2). In the studies described in this thesis, the focus is on four MAPKs: extracellular signal-regulated protein kinase (ERK) 1/2; c-Jun N-terminal kinase (JNK, or stress-activated protein kinases); p38 and ERK5 (or BMK1, big mitogen-activated protein kinase 1), since these four classes of MAPK regulate crucial cellular functions such as survival, proliferation, differentiation and apoptosis (Nithianandarajah-Jones *et al.*, 2012). As a consequence, dysfunction of the MAPK pathways can contribute to tumourigenesis. There are a few studies that suggest alteration of the MAPK signalling is a contributing factor in PC, yet the details of the mechanisms by which MAPKs are regulated in PC are still not fully understood (Tanaka *et al.*, 2003, Uzgare *et al.*, 2003, Kinkade *et al.*, 2008).

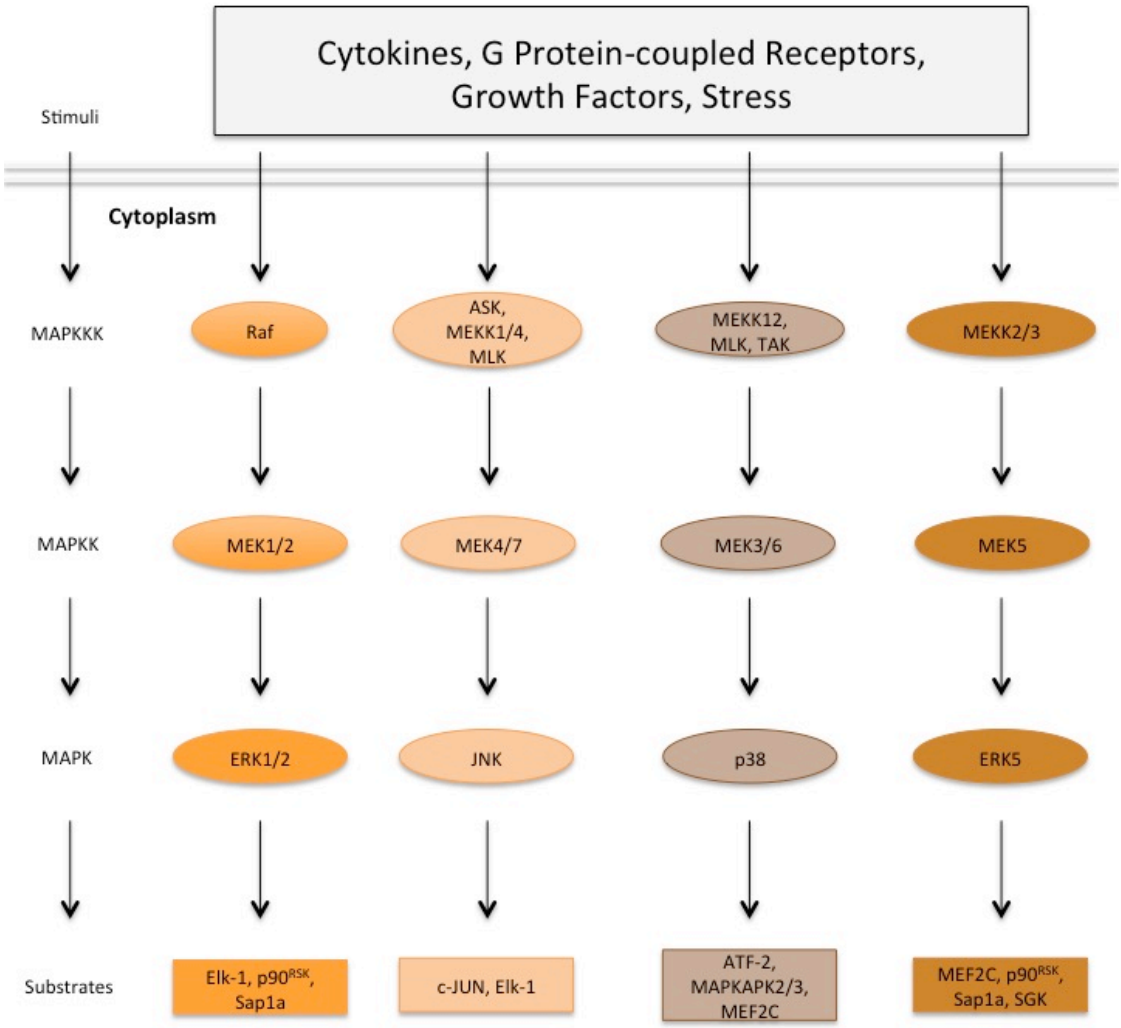


Figure 1.2 Overview of the mitogen-activated protein kinase (MAPK) signalling pathways

Upon stimulation by mitogens such as cytokines, growth factors or stress, the MAPK signalling cascades are initiated by phosphorylation of a mitogen-activated protein kinase kinase kinase (MAPKKK), which phosphorylates a downstream mitogen-activated protein kinase kinase (MAPKK) which subsequently phosphorylates and activates a MAPK. The phosphorylation of the respective downstream substrates of each MAPK leads to physiological responses at the cellular level such as apoptosis, angiogenesis, differentiation, proliferation and survival. Arrow-headed lines denote activation. Different pathways are illustrated by different colours with examples of MAPKKK, MAPKK, MAPK and substrates for each pathway.

1.1.5.4 Role of other pathways

The Wnt proteins are a group of glycoproteins which regulate many aspects of cell behaviour including proliferation, migration, differentiation, survival and polarity by signalling in both β-catenin dependent and β-catenin independent mechanisms (Anastas and Moon, 2013). It has been recognised that abnormal Wnt signalling is involved in many human cancers including PC (Karim *et al.*, 2004, Verras and Sun, 2006, Whitaker *et al.*, 2008, Anastas and Moon, 2013).

The ETs are a family of three small peptides, which exert their biological effects such as apoptosis, mitogenesis and angiogenesis by binding to ET receptors on the cell surface (Battistini *et al.*, 1993, Nelson *et al.*, 2003). The ET axis is known to have a role in cancer (including PC) development and progression, partly through its interaction with other signalling pathways including PI3K/Akt and MAPKs (Pirtskhalaishvili and Nelson, 2000, Bagnato *et al.*, 2011).

HSPs are proteins produced by cells in response to stress to provide a protective measure against cell damage (Feder and Hofmann, 1999, Kregel, 2002). The role of HSPs in cancer is becoming more and more important in determining clinical outcomes (Cornford *et al.*, 2000, Ciocca and Calderwood, 2005).

Crosstalk among these key pathways is thought to be critical in driving carcinogenesis and the development of treatment-resistant disease (Carracedo and Pandolfi, 2008, Bagnato *et al.*, 2011, Carver *et al.*, 2011). In PC, it has been shown that there is a complex crosstalk between AR and other signalling pathways (Lonergan and Tindall, 2011). For example AR can be activated through MAPK or PI3K/Akt signalling pathways (Culig *et al.*, 1994, Abreu-Martin *et al.*, 1999, Peterziel *et al.*, 1999, Sarker *et al.*, 2009). In addition, crosstalk between PI3K/Akt and MAPK signalling pathways also plays a vital role. For example, Akt has been proposed to activate JNK and p38 and inhibit Raf (Unni *et al.*, 2005, Shahabuddin *et al.*, 2006, Belfiore and Malaguarnera, 2011). ERK has also been proposed to inhibit TSC (White and Sharrocks, 2010, Beauchamp and Platanias, 2013) (Figure 1.3).

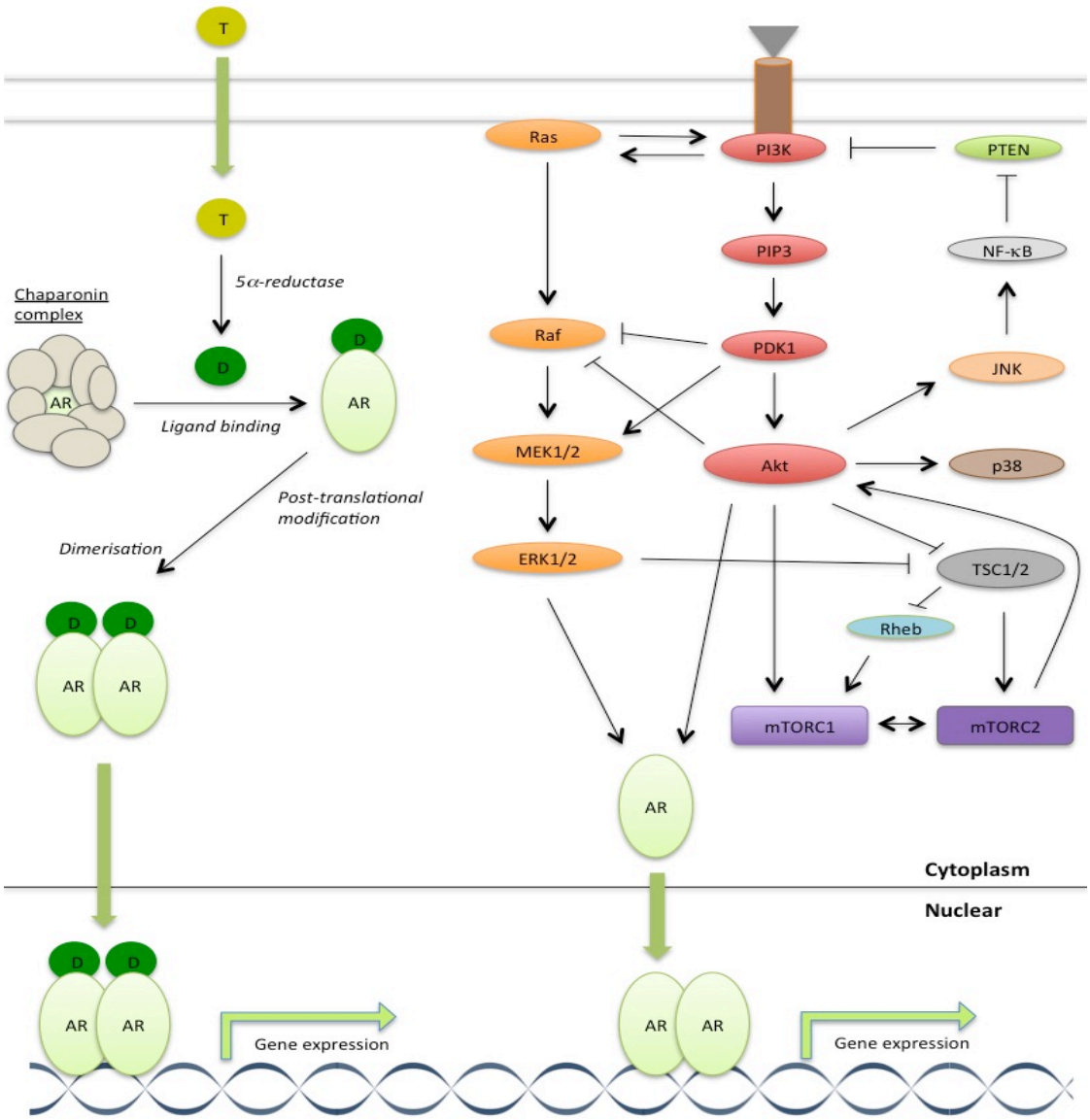


Figure 1.3 Crosstalk network between androgen receptor (AR), PI3K/Akt and MAPK signalling pathways

After passively diffusing into the cytoplasm, testosterone (T) is metabolised to DHT (D). DHT then binds to the AR leading to the physiological activation of the AR signalling pathway. Upon activation, both MAPK and PI3K/Akt pathways can activate AR in the absence of DHT as reviewed by Lonergan *et al* (Lonergan and Tindall, 2011). Complicated crosstalk exists between PI3K/Akt and ERK (Unni *et al.*, 2005, Grant, 2008, White and Sharrocks, 2010, Chappell *et al.*, 2011), JNK (Chen *et al.*, 2002a, Carracedo and Pandolfi, 2008, Belfiore and Malaguarnera, 2011) and p38 signalling pathways (Shahabuddin *et al.*, 2006). Different signalling pathways are colour coded, arrow-headed lines denote activation and bar-headed lines denote inhibition.

1.1.6 Cell lines as tools to examine prostate cancer

Commonly used cultured PC cell lines are derived from metastatic lesions. The most frequently used cell lines are LNCaP, PC3, DU145 and their derivations (Sobel and Sadar, 2005). All of these are derived from metastatic lesions of white males (Sobel and Sadar, 2005). The PC3 and DU145 cell lines are androgen-independent (Sobel and Sadar, 2005). In contrast, the LNCaP cell line is androgen-sensitive (Sobel and Sadar, 2005). Each of the cell lines have shown their ability to grow and proliferate *in vivo* in terms of xenograft tumour formation and metastasis (Sobel and Sadar, 2005).

Since differences exist between each cell line with respect to the original progression, metastasis and AR sensitivity of the individual tumours from which they were derived, research using a selection of such different PC cell lines is useful to provide a more comprehensive understanding of PC biology.

It has recently become apparent that AMP-activated protein kinase (AMPK), a key serine/threonine kinase regulating cellular energy homeostasis (Hardie *et al.*, 2012), has anti-proliferative actions, such that it has been considered as a therapeutic target in cancer (Flavin *et al.*, 2011, Zadra *et al.*, 2015). The role of AMPK in cancer progression is poorly characterised, however (Zadra et al., 2015) and in PC in particular, there are still controversies as to whether AMPK activation is beneficial or detrimental (Park *et al.*, 2009, Chhipa *et al.*, 2011, Zadra *et al.*, 2014).

1.2 AMP-activated protein kinase (AMPK)

1.2.1 Structure and protein levels of AMPK

In mammals, AMPK is a heterotrimeric complex of three subunits: α, β, γ, encoded by seven genes, giving rise to α1, α2, β1, β2, γ1, γ2 and γ3 subunit isoforms (Stapleton *et al.*, 1996). The α subunit contains the catalytic serine/threonine protein kinase domain and β and γ are regulatory subunits (Stapleton *et al.*, 1996, Woods *et al.*, 1996a). Different tissues exhibit differential protein levels of the subunit isoforms (Woods *et al.*, 1996a, Thornton *et al.*, 1998, Cheung *et al.*, 2000) (Figure 1.4).

1.2.2 Regulation of AMPK

1.2.2.1 Allosteric regulation

As is suggested by its name, AMPK is allosterically activated by AMP binding to the γ subunit (Carling *et al.*, 1989, Xiao *et al.*, 2011). Furthermore, ADP or AMP binding to the γ subunit prevents dephosphorylation and inactivation of AMPK, whereas ATP inhibits the effects of AMP and ADP (Carling *et al.*, 2012). The γ subunit contains four Cystathionine β synthase (CBS) repeats, which bind to adenosine-containing ligands (Bateman, 1997, Kemp, 2004, Scott *et al.*, 2004, Ignoul and Eggermont, 2005).

1.2.2.2 Phosphorylation and dephosphorylation

Phosphorylation of AMPK at Thr172 in the α subunit is the principal mechanism that activates AMPK activity (Hawley *et al.*, 1996, Stein *et al.*, 2000). Three AMPK Thr172 kinases have been described, including liver kinase B1 (LKB1) (or serine/threonine kinase 11), calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2) and transforming growth factor beta activated kinase 1 (TAK1) (or mitogen activated protein kinase kinase kinase 7, MAP3K7 or MEKK7) (Hardie, 2011b). LKB1 has been reported to be a constitutive AMPK kinase

(Sakamoto *et al.*, 2004), that supports AMPK activation upon increases in the ratio of AMP:ATP or ADP:ATP. Alternatively, AMPK can be activated by increases in cellular Ca^{2+} in cells that express CaMKK2 independent of changes in adenine nucleotide ratios (Hawley et al., 2005, Hurley et al., 2005, Woods et al., 2005). The physiological relevance of the putative AMPK kinase TAK1, a downstream kinase activated by cytokines remains uncertain (Herrero-Martin *et al.*, 2009). Although not yet fully characterised, studies have indicated that the metal-dependent protein phosphatase family predominantly catalyse AMPK Thr172 dephosphorylation (Carling *et al.*, 2012). It has been suggested that the specific phosphatases that dephosphorylate Thr172 and inactivate AMPK may, however, be dependent on the cell type and stimulus involved (Carling et al., 2012). Furthermore, AMPK Thr172 phosphorylation and activity have been reported to be inhibited by phosphorylation at Ser485/Ser491 in the $\alpha1/\alpha2$ subunit respectively (Hurley *et al.*, 2006). Increased $\alpha1$-Ser485/$\alpha2$-Ser491 phosphorylation has been reported to be stimulated by either autophosphorylation or direct phosphorylation by Akt or protein kinase A (PKA), which may prevent over-activation of AMPK (Hurley *et al.*, 2006) (Figure 1.5).

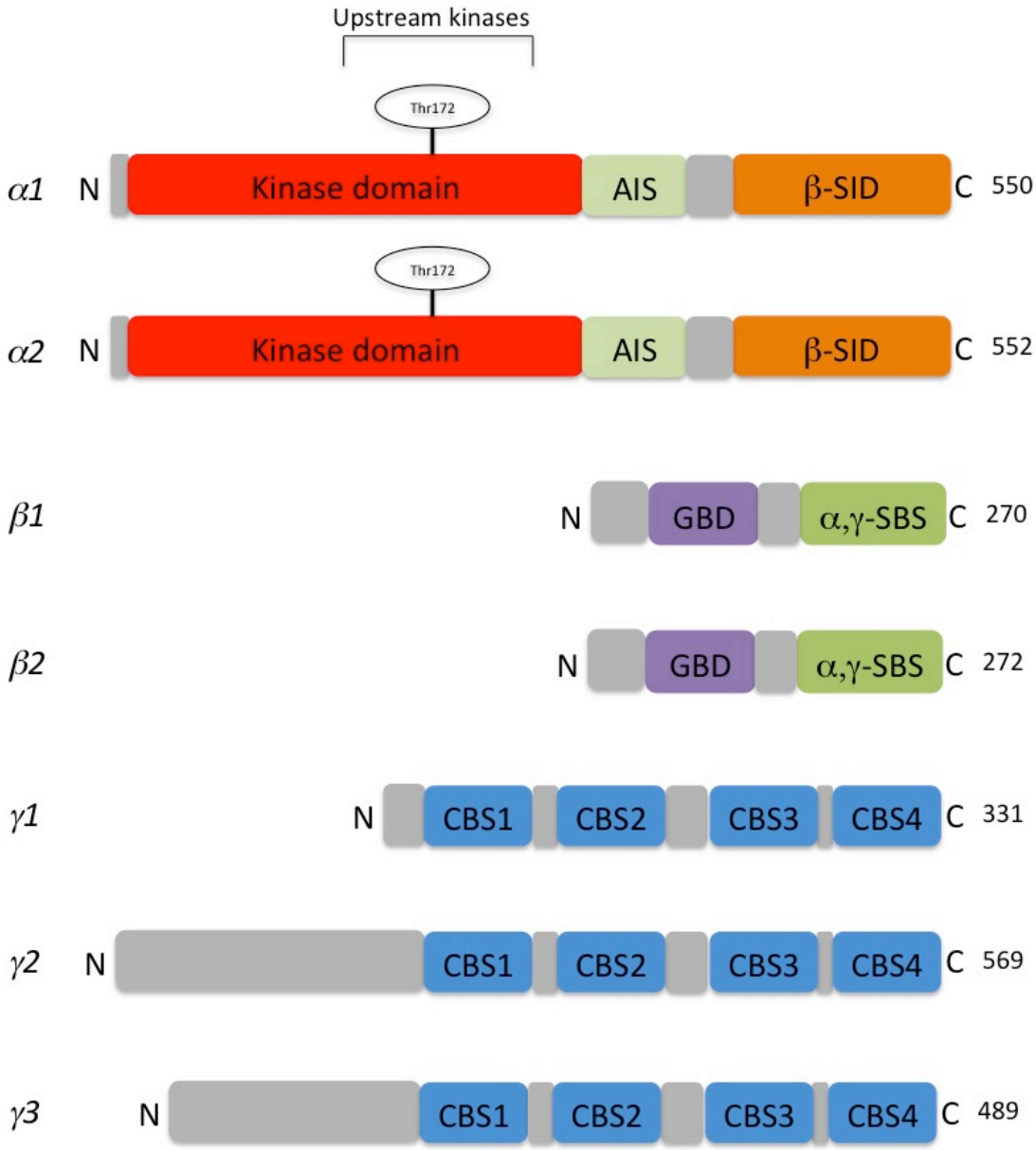


Figure 1.4 Subunit isoforms of AMPK

Schematic representation of AMPK subunit isoform domain structure. N: N-terminus, C: C-terminus. AIS: auto inhibitory sequence; α, γ-SBS: α and γ subunit interacting sequence; β-SID: β subunit interacting domain; CBS: cystathionine β synthase domain; GBD: glycogen binding domain. The site of activating phosphorylation by upstream kinases (Thr172) is shown and the length of each subunit (in amino acids) is shown to the right of the C-terminus in each case.

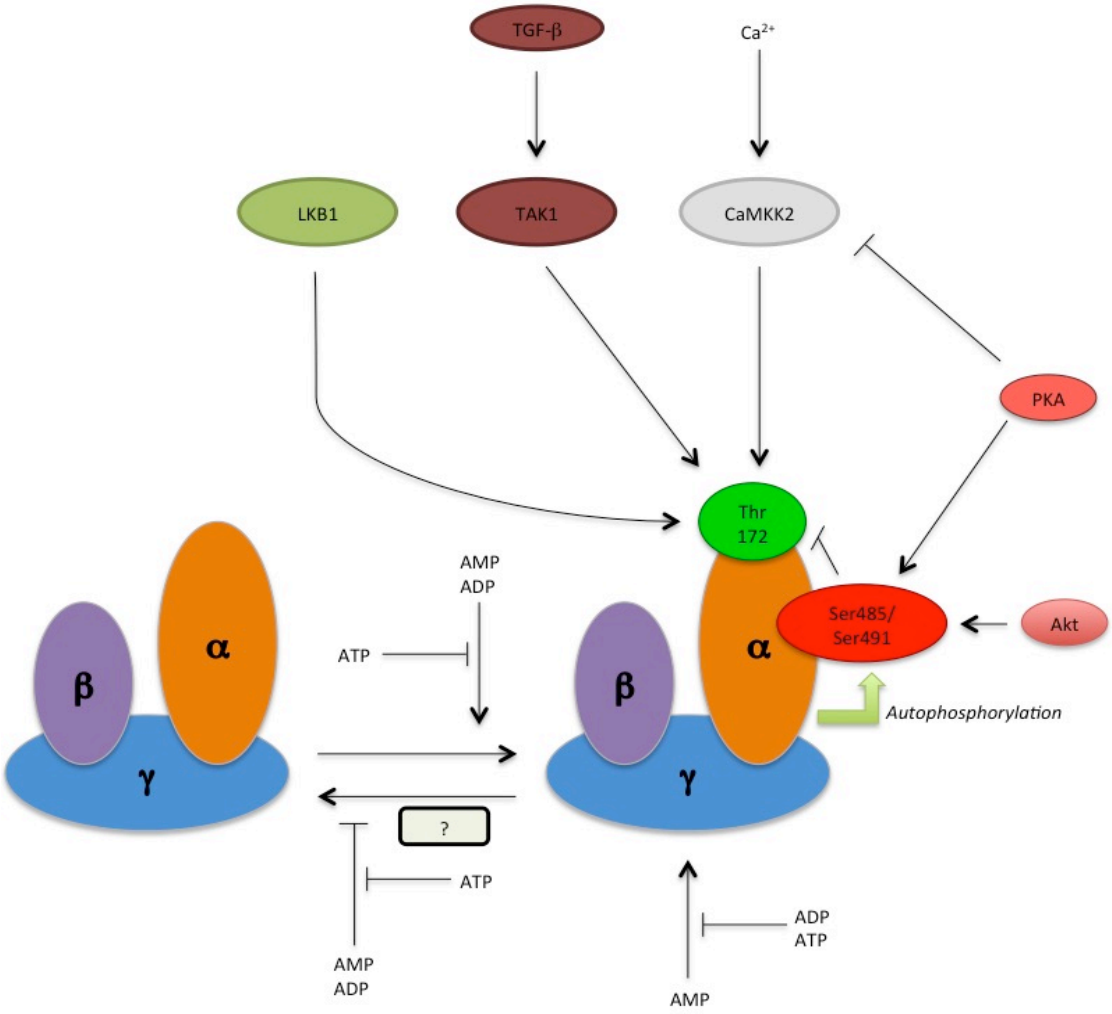


Figure 1.5 Regulation of AMPK activity

The phosphorylation and dephosphorylation of AMPK can be altered by cellular levels of AMP, ADP and ATP, whereby increased AMP or ADP binding to AMPK γ allosterically activates AMPK and promotes liver kinase B1 (LKB1)-mediated phosphorylation, inhibiting dephosphorylation by protein phosphatases. Three Thr172 kinases LKB1, transforming growth factor (TGF)-β activated protein kinase 1 (TAK1) and calcium/calmodulin dependent protein kinase kinase 2 (CaMKK2) have been identified, with LKB1 activity shown to be constitutive and CaMKK2 activated by increasing Ca^{2+}. TAK1 has yet to be shown to be a Thr172 kinase *in vivo*. The α1-Ser485/α2-Ser491 site acts as an inhibitory site, which can either be autophosphorylated by AMPK itself or phosphorylated by Akt or protein kinase A (PKA). An as yet unidentified protein phosphatase catalyses the dephosphorylation of AMPK. Arrow-headed lines denote activation and bar-headed lines denote inhibition.

1.2.3 Activators of AMPK

Physiologically, AMPK is activated by metabolic stresses that increase the AMP:ATP ratio such as hypoxia, ischaemia, exercise (in muscle) and glucose deprivation (Hardie, 2007). In addition, the adipocytokine adiponectin has been demonstrated to activate AMPK in a number of tissues (Yamauchi *et al.*, 2002, Yamauchi *et al.*, 2003, Ouchi *et al.*, 2004, Shibata *et al.*, 2004). 5-aminoimidazole-4-carboxamide-1-β-D- ribofuranoside (AICAR) is a widely used pharmacological activator that works as an adenosine analogue (Hardie, 2011b). It is phosphorylated into the AMP-mimetic ribotide 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP) by adenosine kinase after being taken into cells by adenosine transporters (Hardie, 2011b). AICAR therefore stimulates allosteric activation of AMPK independent of changes in the adenine nucleotide ratio and promotes Thr172 phosphorylation by upstream kinases. WS070117 is also an activator of AMPK that acts as an adenosine analogue (Lian et al., 2011). In addition to these AMP mimetics, A769662 has been used in a number of cell types to directly activate AMPK. A769662 does not influence adenine nucleotide levels but is a specific activator, which not only directly activates AMPK but also inhibits dephosphorylation at Thr172 without directly stimulating the upstream kinases of AMPK (Goransson et al., 2007). A769662 activates AMPK complexes containing the β1 subunit, without affecting AMPK complexes containing β2 subunits (Scott et al., 2008). Due to its high specificity and significantly lower EC_{50}, it is able to initiate AMPK activation at a relatively lower concentration than ribofuranoside or metformin, discussed below (Cool et al., 2006). The subunit isoform specificity of A769662 suggests that compounds similar to A769662 may provide powerful molecular tools for targeted therapeutic approaches in the future.

In addition to these experimental tools to manipulate AMPK activity, a number of existing therapeutics has been demonstrated to activate AMPK. Although the mechanism of action is not yet fully understood, it is believed that part of the pharmacological effects of the hypoglycaemic drug metformin is through activation of AMPK (Zhou *et al.*, 2001, Zhang *et al.*, 2012, Hardie, 2013). Several lines of evidence also suggest that metformin has an anti-cancer effect in several different types of cancer in both *in vitro* and *in vivo* studies (Ben Sahra *et al.*,

2010a, Foretz *et al.*, 2010, Ferla *et al.*, 2012, Luo *et al.*, 2012, Menendez *et al.*, 2012, Cerezo *et al.*, 2013, Duo *et al.*, 2013, Habibollahi *et al.*, 2013, Storozhuk *et al.*, 2013, Hadad *et al.*, 2014, Malaguarnera *et al.*, 2014). Several mechanisms may contribute to this effect of metformin, including altering cellular metabolism and energy homeostasis as well as regulating the cell cycle (Martin and Marais, 2012, Choi and Park, 2013, Pierotti *et al.*, 2013, Leone *et al.*, 2014). Clinical evidence also suggests that treatment of metformin in people with and without diabetes reduces the risk of developing cancer and also leads to a better outcome in many malignant conditions including PC (Evans *et al.*, 2005, Libby *et al.*, 2009, Wright and Stanford, 2009, Ben Sahra *et al.*, 2010b, Decensi *et al.*, 2010, Azoulay *et al.*, 2011, Hadad *et al.*, 2011, Nobes *et al.*, 2012, Ruiter *et al.*, 2012, Zhang *et al.*, 2013, Anwar *et al.*, 2014). Using AMP-insensitive mutants of AMPK, evidence has shown that metformin activates AMPK by inhibiting the mitochondrial electron transport chain, thereby increasing AMP (Hawley *et al.*, 2010).

In addition to the biguanide (metformin) class of anti-diabetic drugs, the thiazolidinedione (rosiglitazone and pioglitazone) class of anti-diabetic drugs have also been demonstrated to activate AMPK, although again, their actions are not mediated exclusively through AMPK (Zhou *et al.*, 2001, Fryer *et al.*, 2002). Recently, salicylate has been reported to activate AMPK directly in a similar manner to A769662, specifically stimulating complexes containing β1 subunit isoforms (Hawley *et al.*, 2012). It has also been reported that statins, drugs used to lower endogenous cholesterol synthesis in those at risk of cardiovascular diseases, activate AMPK (Sun *et al.*, 2006). Interestingly, a number of xenobiotics found in traditional herbal medicines also activate AMPK including galegine, berberine and hispidulin (Lee *et al.*, 2006, Mooney *et al.*, 2008, Lin *et al.*, 2010).

1.2.4 Physiological function of AMPK

AMPK is a key regulator of metabolism by maintaining energy homeostasis. It senses the energy status by measuring the AMP:ATP ratio in the cytoplasm and transfers the signal to modulate ATP production and consumption (Hardie, 2011b). In order to maintain energy homeostasis, AMPK responds to increased

AMP:ATP or ADP:ATP by stimulating catabolic pathways and inhibiting anabolic pathways, thereby normalising cellular ATP (Hardie, 2011b). AMPK phosphorylates and inhibits many metabolic enzymes such as acetyl-CoA carboxylase (ACC) 1, hydroxymethylglutaryl-CoA reductase (HMGR), glycerol phosphate acyl transferase and glycogen synthase (Hardie, 2007b, Hardie, 2011b). It can also down-regulate RNA synthesis by phosphorylating RNA polymerase I associated transcription factor (Hoppe *et al.*, 2009). Consequently, fatty acid synthesis, isoprenoid synthesis, triglyceride, phospholipid synthesis, glycogen synthesis and ribosomal RNA synthesis are inhibited, which are critical for rapid cell growth and proliferation (Hardie, 2007b, Hoppe *et al.*, 2009, Hardie, 2011b). In contrast, AMPK activation enhances mitochondrial biogenesis, autophagy and mitophagy (Hardie, 2011a). AMPK also stimulates fatty acid oxidation due to the inhibition of ACC leading to reduce formation of malonyl-CoA (Kudo *et al.*, 1995). Malonyl-CoA inhibits carnitine palmitoyl transferase in the mitochondrial membrane, which is the committed step of fatty acid oxidation (McGarry *et al.*, 1978). Therefore AMPK activation inhibits fatty acid synthesis and stimulates fatty acid oxidation at the same time. In addition, AMPK is involved in cell polarity maintenance (Hardie, 2011a).

1.2.4.1 Regulation of acetyl-CoA carboxylase by AMPK

Acetyl-CoA carboxylase (ACC) is an important regulatory enzyme in fatty acid synthesis, catalysing the carboxylation of acetyl-CoA to malonyl-CoA (McGarry and Foster, 1980, Cook *et al.*, 1984, Zierz and Engel, 1987). There are two isoforms of ACC, whereby ACC1 is involved in fatty acid synthesis and ACC2 is associated with mitochondria and thought to regulate fatty acid oxidation (Abu-Elheiga *et al.*, 2000, Abu-Elheiga *et al.*, 2001). Both ACC isoforms can be phosphorylated and inhibited by AMPK *in vitro* and *in vivo* (Davies *et al.*, 1992, Winder and Hardie, 1996).

1.2.4.2 Regulation of hydroxymethylglutaryl-CoA reductase by AMPK

Hydroxymethylglutaryl-CoA reductase (HMGR) is an important regulatory enzyme, which synthesises mevalonate from hydroxymethylglutaryl-CoA.

Mevalonate synthesis is required for isoprenoid and cholesterol biosynthesis (Holstein and Hohl, 2004). AMPK inhibits HMGR by phosphorylating Ser872 at the active site, thereby decreasing enzyme catalytic efficiency (Clarke and Hardie, 1990, Istvan and Deisenhofer, 2000, Burg and Espenshade, 2011).

1.2.4.3 Regulation of hormone-sensitive lipase by AMPK

Hormone-sensitive lipase (HSL) plays a vital role in fatty acid mobilisation by hydrolysing triglycerides to fatty acids and glycerol (Holm, 2003). Studies have suggested that AMPK can phosphorylate HSL, so that lipolysis is inhibited (Carling and Hardie, 1989, Corton et al., 1995, Garton et al., 1989, Sullivan et al., 1994).

1.2.4.4 Regulation of mammalian target of rapamycin (mTOR) by AMPK

AMPK activation also inhibits the mTOR pathway by phosphorylating TSC2 and mTORC1 subunit Raptor (Inoki et al., 2003, Gwinn et al., 2008). The mTOR pathway itself is responsible for mRNA translation and ribosomal biogenesis (Hay and Sonenberg, 2004). Thus, protein synthesis is inhibited upon AMPK activation (Hardie, 2011b).

1.2.4.5 Regulation of other metabolic targets by AMPK

Glycogen is the major form of stored carbohydrate in mammals. AMPK activation has been reported to inhibit glycogen synthesis by phosphorylation of glycogen synthase (Carling and Hardie, 1989, Jorgensen *et al.*, 2004). Mitochondria plays a key role in energy homeostasis, signalling, apoptosis and metabolism (Hock and Kralli, 2009). Multiple reports have shown that AMPK activation stimulates mitochondrial biogenesis, thereby improving oxidative metabolism and ATP synthesis (Zong *et al.*, 2002, Hardie *et al.*, 2012).

1.2.5 Crosstalk between AMPK and PI3K/Akt signalling pathways

It is becoming more interesting that AMPK could influence the PI3K/Akt/mTOR pathway via crosstalk (Shackelford and Shaw, 2009, Song *et al.*, 2012, Beauchamp

and Platanias, 2013, Kim and He, 2013, Martin and Marais, 2013). Studies have shown that AMPK is an upstream kinase, which could inhibit mTORC1 activity through a dual mechanism either by phosphorylating the TSC2 or the raptor subunits *in vitro* (Inoki *et al.*, 2003, Cheng *et al.*, 2004, Gwinn *et al.*, 2008). Details of the interaction between AMPK and PI3K/Akt/mTOR pathways have been thoroughly reviewed (Shackelford and Shaw, 2009, Inoki *et al.*, 2012, Beauchamp and Platanias, 2013, Kim and He, 2013). Unc-51 like autophagy-activating kinase 1 has been reported as an important kinase participated in AMPK/mTOR feedback loop in maintaining energy homeostasis (Egan *et al.*, 2011, Kim *et al.*, 2011, Dunlop and Tee, 2013) (Figure 1.6).

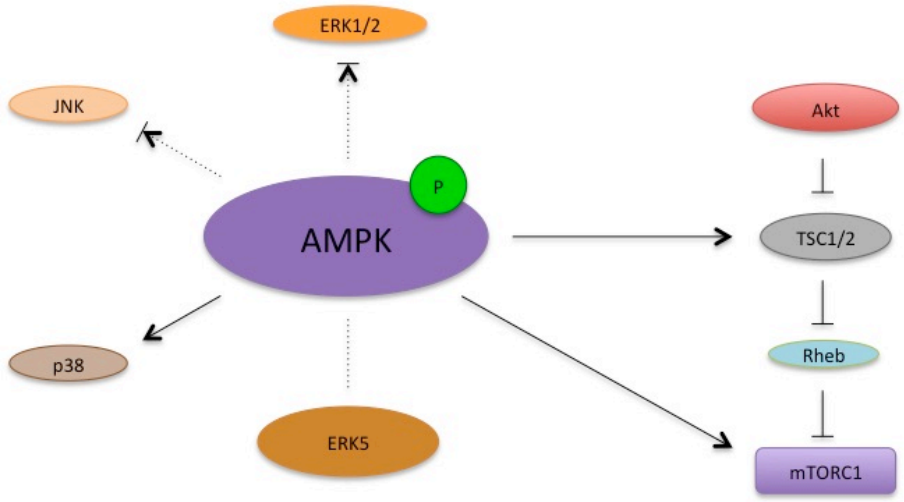


Figure 1.6 Crosstalk between AMPK, MAPK and Akt signalling pathways
The signalling crosstalk network between AMPK and MAPK (Young *et al.*, 2009, Yun *et al.*, 2009, Kim and He, 2013, Martin and Marais, 2013, Chaube *et al.*, 2015), Akt (Kim *et al.*, 2011) is not fully understood. Dotted lines denote proposed effects. Arrow-headed lines denote activation and bar-headed lines denote inhibition.

1.2.6 Crosstalk between AMPK and mitogen-activated protein kinase (MAPK) signalling pathways

AMPK activation has been reported to regulate MAPK signalling pathways, however the mechanisms are not fully understood (Chen *et al.*, 2002b, Li *et al.*, 2005, Schulz *et al.*, 2008, Young *et al.*, 2009). Berberine-stimulated AMPK activation was shown to reduce phosphorylation (and activation) of ERK1/2, JNK and p38 in macrophages (Jeong *et al.*, 2009). Similarly, AMPK activation was associated with inhibition of ERK1/2 phosphorylation in fibroblasts (Du *et al.*, 2008). Activation of AMPK by AICAR has also been demonstrated to down-regulate phosphorylation of ERK1/2 *in vitro* and *in vivo* (Motobayashi *et al.*, 2009, Meng *et al.*, 2011) (Figure 1.6). Therefore, it has been proposed that AMPK is an upstream regulator of ERK1/2 (Turcotte et al., 2005). In contrast, other studies have suggested that AMPK activates ERK1/2 *in vitro* (Chen *et al.*, 2002b, Sweadner, 2008) (Figure 1.6).

Schulz and colleagues found that AMPK activation was associated with inhibition of JNK phosphorylation *in vitro* (Schulz *et al.*, 2008). It has also been proposed that there is a feedback loop between JNK and AMPK. In DU145 cells (which lack LKB1), JNK activation increased AMPK activity whereas in DU145 cells transfected with LKB1 however, AMPK inhibits JNK activation (Yun *et al.*, 2009). In contrast, other studies suggest AMPK activation increases JNK activation *in vitro* using hepatoma (FTO2B) cells (Meisse *et al.*, 2002). Lee and co-workers have also reported that AMPK activation by AICAR led to activation of JNK in HepG2 cells (Lee *et al.*, 2008).

Data concerning the regulation of p38 and ERK5 by AMPK are more sparse, although studies have suggested that AMPK up-regulates p38 phosphorylation both *in vitro* and *in vivo* (Li *et al.*, 2005, Han *et al.*, 2009, Meng *et al.*, 2011). Moreover, AMPK has been reported to be a vital upstream regulator for ERK5 signalling in endothelial cells (Young *et al.*, 2009).

1.2.7 Role of AMPK in cancer

AMPK is now considered to be a potential therapeutic target for metabolic disorders as well as diseases based on cellular proliferation, including type 2 diabetes mellitus, metabolic syndrome, atherosclerosis and cancer (Motoshima *et al.*, 2006, Hardie, 2007a, Rutter and Leclerc, 2009, Viollet *et al.*, 2009, Hardie, 2011a, Hardie and Alessi, 2013, Pierotti *et al.*, 2013). It has also been suggested that the anti-inflammatory properties of AMPK activation may also beneficial for chronic inflammatory diseases and cancer (Salt and Palmer, 2012, Dandapani and Hardie, 2013). The association between activation of AMPK and reduced proliferation has been demonstrated in many different types of cancer cell lines. Recent studies in this field have included not only cell lines derived from many solid tumours such as lung cancer, breast cancer, bladder cancer, ovarian cancer, renal cancer, malignant melanoma, pancreatic cancer, thyroid cancer, glioblastoma, colon cancer and PC, but also cell lines derived from haemotological malignancies such as acute lyphoblastic leukaemia, mantle cell lymphoma and acute myeloid leukaemia (Lee *et al.*, 2012, Vakana *et al.*, 2012, Zheng *et al.*, 2012).

It is known that AMPK signalling is linked to at least two tumour suppressors, LKB1 and TSC2 (Hawley *et al.*, 2003, Woods *et al.*, 2003, Xiang *et al.*, 2004). Research suggests that AMPK is responsible for the tumour suppressing effects of LKB1 (Hardie, 2011b). Evidence that AMPK has protective effects against tumour development are based on several possible mechanisms: a) treatment with different AMPK activators in animal models can delay tumour development (Huang *et al.*, 2008); b) down-regulation of AMPK activation in certain cancers has been observed, possibly due to loss of LKB1 (Hawley *et al.*, 2003, Zheng *et al.*, 2009); c) phosphorylation at Ser485/Ser491 of AMPK α subunits by Akt (or protein kinase B) down-regulates AMPK activity in tumours which contain hyper-activated Akt, which in turn, inhibits the phosphorylation at Thr172 by LKB1 (Horman *et al.*, 2006); d) TSC2 phosphorylation by AMPK inhibits the PI3K/AKT/mTOR pathway (Li *et al.*, 2004, Xiang *et al.*, 2004, Hardie, 2011a); e) AMPK activation reduces the synthesis of phospholipid for membrane synthesis (Hardie, 2011b).

Although AICAR is limited in clinical use due to its toxicity (Dixon *et al.*, 1991), it significantly reduces cell proliferation in HeLa, DU145 and HepG2 cells but not in non-cancer cells dependent on energy status and bioenergetic profile (Jose *et al.*, 2011). Metformin also causes a significant repression of proliferation in breast cancer cell lines (Phoenix *et al.*, 2009). It has also been reported that people with diabetes treated with metformin have a lower incidence of cancer (including PC) than their counterparts treated with other hypoglycaemic drugs (Evans *et al.*, 2005). Salicylate and metformin have both been reported to suppress PC survival in *ex vivo* studies (O'Brien *et al.*, 2015). Activation of AMPK by ionizing radiation (IR) has also been reported to enhance the cytotoxic effects of IR in cancer cells (Sanli *et al.*, 2010). However, whether AMPK activation is important as a target for cancer therapy remains to be tested (Hardie, 2011a). In contrast to the notion that activation of AMPK is beneficial with cancer-suppressive effects, it has been proposed that AMPK may function through NADPH regulation to promote tumour cell survival under conditions of energy deprivation (Jeon *et al.*, 2012). Therefore, further investigation of the role of AMPK in cancer is warranted.

1.2.8 Potential AMPK regulation of prostate cancer signalling

At the beginning of these studies, it had been reported that AMPK activation in PC cell lines by either AICAR or thiazolidinedione inhibits cell growth *in vitro* (Xiang *et al.*, 2004) indicating that inhibition of AMPK could lead to an increase of PC proliferation, thus promoting malignancy in terms of migration and growth (Zhou *et al.*, 2009). DU145 cells lack the AMPK kinase LKB1, yet AMPK can surprisingly be activated by AICAR in such cells (Yun *et al.*, 2005), despite LKB1 being considered essential for AICAR-mediated AMPK phosphorylation (Hutber *et al.*, 1997). These interesting results imply that AICAR can activate AMPK in PC cells by LKB1-independent mechanisms. It has been argued that this effect might be a consequence of reactive oxygen species production (Yun *et al.*, 2005, Jose *et al.*, 2011). Moreover, JNK has also been reported to be involved as upstream of AMPK in this pathway (Yun *et al.*, 2005).

Despite these findings, it has also been reported that *in vitro* inhibition of AMPK inhibits PC cell growth, suggesting a proliferative action of AMPK (Park *et al.*, 2009). This argument is particularly interesting when considering an energy-starved microenvironment, which most solid tumours are subjected to. Under such a condition, AMPK is activated and may promote PC cell survival in androgen-independent PC cells *in vitro* (Chhipa *et al.*, 2010). Indeed, immunohistochemistry revealed elevated phosphorylated ACC staining in human PC specimens (Park *et al.*, 2009). In additional, high levels of phosphorylated ACC and phosphorylated AMPK were found in both androgen-sensitive and androgen-independent PC cell lines (including PC3, DU145 and LNCaP) analysed by immunoblotting (Park *et al.*, 2009). As AMPK is activated in an energy-stress environment, whether higher AMPK activation in cancer tissue is contributing to malignancy itself or is indeed a protective reaction against the abnormality has not yet been characterised.

In addition, AMPK activation is implicated in angiogenesis, required for tumour expansion and metastasis. AMPK activation is required for angiogenesis in endothelial cells and down-regulation of AMPK inhibits migration and proliferation of endothelial cells (Nagata *et al.*, 2003, Reihill *et al.*, 2011). Studies have reported that the expression of the activating AMPK Thr172 kinase CaMKK2 is elevated in PC cell lines, especially in response to AR stimulation. As a result, inhibition of the CaMKK2/AMPK pathway could block androgen-stimulated cell migration and growth (Frigo *et al.*, 2011, Massie *et al.*, 2011). Unlike normal cells, cancer cells are more likely to depress the AMPK signalling pathway which would normally lead to reduced cell growth (Carling *et al.*, 2012). It is therefore necessary to further unravel the potential effects of pathways upstream and downstream of AMPK in different types of malignancy and different stages of cancer progression.

1.3 Hypothesis and aims

The primary hypothesis of these studies is that AMPK activation has anti-cancer effects in PC cells. The mechanism underlying these effects may be mediated by reduced MAPK and (or) PI3K/Akt pathway signalling. Specifically, these studies sought to answer the following research questions comparing effects in androgen-independent and androgen-dependent PC cell lines:

1. What AMPK subunit isoforms and AMPK kinases are expressed in PC cells?

2. How do AMPK activators influence PC cell line proliferation, viability and migration?

3. Does AMPK activation influence basal or stimulated MAPK and/or Akt activity in PC cell lines?

Chapter 2. Materials and methods

2.1 Materials

2.1.1 Suppliers of materials

Abcam, Cambridge, UK

A769662 (6,7-Dihydro-4-hydroxy-3-(2'-hydroxy(1,1'-biphenyl)-4-yl)-6-oxo-thieno(2,3-*b*)pyridine-5-carbonitrile)

BDH Laboratory Supplies, Poole, UK

Coomassie brilliant blue G-250

Corning Life Sciences, Tewksbury, MA, USA

12-well tissue culture plates

24-well cell culture insert companion plates

6 cm and 10 cm diameter tissue culture dishes

6-well tissue culture plates

96-well culture plates

Migration chambers

Tissue culture T75 flasks

Cell Signaling Technology, Danvers, MA, USA

Cell Lysis Buffer #7018

PathScan Stress and Apoptosis Signaling Antibody Array Kit (Fluorescent Readout) #12923

Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK

Ammonium persulphate (APS)

Tris base (tris (hydroxymethyl) aminoethane)

Formendium, Hunstanton, Norfolk, UK

Bacterial Agar

Tryptone

Yeast extract powder

GE Healthcare, Little Chalfont, Buckinghamshire, UK

Protein A sepharose beads

Protein G sepharose beads

Life Technologies Ltd, Paisley, UK

Alexa Fluor Dyes

Dulbecco's modified Eagles meda (DMEM)

Foetal calf serum (FCS) (EU origin)

Human EGF

L-glutamine

Lipofectamine

Opti-MEM Reduced serum media

Penicillin/streptomycin

Trypsin

Li-Cor Biosciences, Lincoln, NE, USA

Odyssey blocking buffer

Lonza Group Ltd, Cologne, Germany

Nucleofector Kit L, Kit V

pmaxGFP Vector

Melford Laboratories Ltd, Chelsworth, Ipswich, Suffolk, UK

Dithiothreitol (DTT)

Merck Chemicals Ltd, Nottingham, UK

Compound C

Millipore Limited, Hertfordshire, UK

Akt Inhibitor VIII

BrdU kit

New England Biolabs, Ipswich, MA, USA

Gel loading dye (6 ×)

Prestained protein marker (broad range 6-175 kDa)

PALL Life Sciences, Pensacola, FL, USA

Nitrocellulose transfer membrane, 0.45 μM pore size

Premier International Foods, Cheshire, UK

Dried skimmed milk

Qiagen Ltd, Crawley, West Sussex, UK

AllStars non-silencing siRNA

FlexiTube *PRKAA1* siRNA

Plasmid Maxi Kit

Roche Diagnostic Ltd, Burgess Hill, UK

Proteinase inhibitor cocktail tablets, EDTA-free

WST-1 reagent

Severn Biotech Ltd, Kidderminster, Hereford, UK

Acrylamide: Bisacrylamide (37.5:1; 30% (w/v) Acrylamide)

Sigma-Aldrich Ltd, Gillingham, Dorset, UK

Benzamidine

Bovine serum albumin (BSA)

D-mannitol

Donkey serum

DPX mountant

Ethylenediamine tetraacetic acid (EDTA)

Ethylene glycol-bis (β-amino-ethyleter)-N,N.N',N'-tetraacetic acid (EGTA)

Fish skin gelatin

G418

Haematoxylin

N,N,N',N'-Tetramethylethylenediamine (TEMED)
Paraformaldehyde
Phenylmethylsulphonyl fluoride (PMSF)
Ponceau S stain
Soyabean trypsin inhibitor (SBTI)
Triton X-100
Tween-20

Thermo Scientific, Waltham, MA, USA
Immunomount
ON-TARGETplus *PRKAA1* siRNA
Texas Red-X Phalloidin

Tocris Bioscience, Bristol, UK
PD184352

Toronto Research Chemicals Inc, Ontario, Canada
AICAR (5-aminoimidazole-4-carboxamide-1-β-D- ribofuranoside)

VWR International Ltd, Lutterworth, Leicestershire, UK
Falcon tissue culture 6 cm diameter dishes and plates
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid)

2.1.2 Suppliers of equipment

Beckman Coulter, High Wycombe, UK
Allegra X-12 centrifuge
Multi-purpose scintillation counter LS 6500
Optima XL-80K ultracentrifuge
SW40 rotor

Bibby Scientific Limited, Staffordshire, UK
Genova Life Science spectrophotometer

Bio-Rad Laboratories, Hemel Hempstead, UK

Protein gel casting and Western blotting equipment

BMG Labtech GmbH, Ortenberg, Germany

SPECTROstar Nano microplate reader

Carl Zeiss Ltd, Cambridge, UK

Axiovert 200M confocal microscope

Axiovert 25 inverted fluorescence microscope

Axiovision light microscope

LSM 5 PASCAL Exciter instrument

DJB Labcare Ltd, Lincoln, Buckinghamshire, UK

Hettich Mikro 22R centrifuge

Li-Cor Biosciences, Lincoln, NE, USA

Odyssey Sa Image System

Lonza Group Ltd, Cologne, Germany

Nucleofector II

Nikon UK Limited, Surrey, UK

TE2000 time-lapse microscope

Olympus, Essex, UK

BM50 microscope

Optika Microscopes, Ponteranica, Italy

XDS-1B light microscope

Qimaging, Surrey, BC, Canada

Retiga EXi Fast 1394 digital camera

Thermo Scientific, Waltham, MA, USA

Nanodrop spectrophotometer

2.1.3 Suppliers of cells

American Type Culture Collection, Manassas, VI, USA

DU145, LNCaP, PC3, PC3M cells

PC3 cell lines overexpressing ERK5 (PC3-ERK5-17P-Flag, PC3-ERK5-18R-Flag) and the control cell line (PC3-EmptyVector) were generated by Janis Fleming as previously described in the Leung laboratory (Ramsay *et al.*, 2011).

AMPK α1$^{-/-}$ *AMPK α2*$^{-/-}$ knockout (KO) and wild type (WT) mouse embryonic fibroblasts (MEFs) were a kind gift from Dr Benoit Viollet (Paris, France).

2.1.4 Suppliers of antibodies and conditions of use

2.1.4.1 Antibodies for immunoblotting

Details of primary antibodies (Table 2.1) and secondary antibodies (Table 2.2) used for immunoblotting are supplied below.

2.1.4.2 Antibodies for immunofluorescence microscopy

Details of primary antibodies used for immunofluorescence microscopy are provided below (Table 2.3).

Epitope	**Host species**	**Clonality**	**Dilution**	**Supplier**	**Note**
ACC	Rabbit	Monoclonal	1:1, 000	CST	#3676
ACC1	Sheep	Polyclonal	1:1, 000	Dundee	(CQRDFTVASPAEFVT)
Akt	Mouse	Monoclonal	1:2, 000	CST	#2920
AMPK alpha	Rabbit	Polyclonal	1:1, 000	CST	#2532
AMPK alpha1	Sheep	Polyclonal	1:1, 500	Dundee	(TSPPDSFLDDHHLTR) (Woods *et al.*, 1996b)
AMPK alpha2	Sheep	Polyclonal	1:1, 000	Dundee	(MDDSAMHIPPGLKPH) (Woods *et al.*, 1996b)
AMPK beta1N	Sheep	Polyclonal	1:180	Dundee	(KTPRRDSSGGT)
AMPK beta2	Sheep	Polyclonal	1:1, 000	Dundee	(CSVFSLPDSKLPGDK)
AMPK gamma1	Sheep	Polyclonal	1:1, 000	Dundee	(PENEHSQETPESNS) (Cheung *et al.*, 2000)
AMPK gamma2C	Sheep	Polyclonal	1:1, 000	Dundee	(CLTPAGAKQKETETE)
AMPK gamma3C	Sheep	Polyclonal	1:1, 000	Dundee	(CLSPAGIDPSGPEKI)
CaMKK2	Mouse	Monoclonal	1:100	Dundee	4H8
c-Myc	Mouse	Monoclonal	1:1, 000	Santa Cruz	sc-40
ERK1/2	Mouse	Polyclonal	1:1, 000	CST	#9102
ERK5	Mouse	Polyclonal	1:1, 000	CST	#3372
GAPDH	Mouse	Monoclonal	1:80, 000	Ambion	AM4300

GFP	Rabbit	Polyclonal	1:3, 000	Abcam	ab290
HA-probe (F7)	Mouse	Monoclonal	1:1, 000	Santa Cruz	sc-7392
HA.11 (16B12)	Mouse	Monoclonal	1:1, 000	Covance	MMS-101P
JNK	Rabbit	Polyclonal	1:1, 000	CST	#9252
LKB1	Rabbit	Monoclonal	1:1, 000	CST	#3050
MEK5	Mouse	Monoclonal	1:4, 000	BD	610957
p-ACC (Ser79)	Rabbit	Polyclonal	1:1, 000	CST	#3661
p-Akt (Ser473)	Rabbit	Monoclonal	1:1, 000	CST	#4058
p-Akt (Thr308)	Rabbit	Monoclonal	1:1, 000	CST	#13038
p-AMPK alpha (Thr172)	Rabbit	Monoclonal	1:1, 000	CST	#2535
p-AMPK alpha1 (Ser485)	Rabbit	Monoclonal	1:1, 000	CST	#2537
p-AMPK alpha1/2	Rabbit	Polyclonal	1:1, 000	CST	#4185
p-ERK1/2	Mouse	Monoclonal	1:1, 000	CST	#9106
p-ERK5	Rabbit	Polyclonal	1:500	CST	#3371
p-ERK5	Rabbit	Polyclonal	1:1, 000	Millipore	07-507
p-ERK5	Goat	Polyclonal	1:200	Santa Cruz	sc-16564
p-JNK	Rabbit	Polyclonal	1:1, 000	CST	#9251
p-JNK	Mouse	Monoclonal	1:2, 000	CST	#9255
p-MEK5	Rabbit	Polyclonal	1:1, 000	Millipore	PK-1000
p-MEK5	Rabbit	Polyclonal	1:2, 000	Santa Cruz	sc-135702

p-p38	Rabbit	Monoclonal	1:1, 000	CST	#4511
p-p38	Mouse	Monoclonal	1:2, 000	CST	#9216

Table 2.1 Primary antibodies for immunoblotting

CST: Cell Signaling Technology, Dundee: Antibodies provided by Prof D Grahame Hardie, University of Dundee, Dundee, UK.

Linked molecule	Epitope	Host species	Dilution	Manufacture	Note
Alexa Fluor 680 (Red)	Sheep IgG	Donkey	1:2, 000	Life Technologies	#A21102
IRDye 680LT (Red)	Mouse IgG	Donkey	1:10, 000	Li-Cor	926-68022
IRDye 680LT (Red)	Rabbit IgG	Donkey	1:10, 000	Li-Cor	926-68023
IRDye 680LT (Red)	Goat IgG	Donkey	1:10, 000	Li-Cor	926-68024
IRDye 800CW (Green)	Mouse IgG	Donkey	1:10, 000	Li-Cor	926-32212
IRDye 800CW (Green)	Rabbit IgG	Donkey	1:10, 000	Li-Cor	926-32213

Table 2.2 Secondary antibodies for immunoblotting

Epitope	Host species	Clonality	Dilution	Manufacture	Note
AMPK alpha1	Sheep	Polyclonal	1:100	Dundee	α1 (2nd)
Sheep IgG	Donkey	Polyclonal – conjugated to Alexa Fluor 488 (Green)	1:400	Life Techonologies	#A-11015

Table 2.3 Primary antibodies for immunofluorescence microscopy

Dundee: Antibodies provided by Prof D Grahame Hardie, University of Dundee, Dundee, UK.

2.1.5 Solutions

2YT medium (pH 7.0)

0.5% (w/v) NaCl

1% (w/v) yeast extract

1.5% (w/v) tryptone

100 μg/mL ampicillin

2% (w/v) agar

Bradford's reagent

35 mg/L coomassie brilliant blue

5% (v/v) ethanol

5.1% (v/v) orthophosphoric acid

Bradford's reagent was filtered and stored in the dark

Buria-Ertani medium

0.5% (w/v) yeast extract

1% (w/v) NaCl

1% (w/v) tryptone

Immunofluorescence (IF) buffer

0.1% (v/v) donkey serum

0.2% (w/v) fish skin gelatin

0.9 mM KH_2PO_4

1.7 mM KCl

5 mM Na_2HPO_4

85 mM NaCl

Lysogeny Broth (LB) medium (pH 7.5)

0.5% (w/v) yeast extract

1% (w/v) tryptone

100 μg/mL ampicillin

171.2 mM NaCl

Lysis Buffer

0.1 mM benzamidine

0.1 mM PMSF

1% (v/v) Triton-X-100

1 mM DTT

1 mM EDTA

1 mM EGTA

1 mM Na_3VO_4

1 mM $Na_4P_2O_7$

250 mM mannitol

50 mM NaF

50 mM Tris-HCl, pH 7.4 at 4°C

5 μg/mL SBTI

Phosphate-buffered saline (PBS) pH7.2

0.9 mM KH_2PO_4

1.7 mM KCl

5 mM Na_2HPO_4

85 mM NaCl

Phosphate-buffered saline + Tween 20 (PBST)

0.1% (v/v) Tween 20

0.9 mM KH_2PO_4

1.7 mM KCl

5 mM Na_2HPO_4

85 mM NaCl

Ponceau S stain

0.2% (w/v) ponceau-S

1% (v/v) acetic acid

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) running buffer

0.1% (w/v) SDS

190 mM glycine

62 mM Tris base

4 × SDS-PAGE sample buffer

0.4% (w/v) bromophenol blue

200 mM DTT

200 mM Tris-HCl, pH 6.8

40% (v/v) glycerol

8% (w/v) SDS

SOC medium (pH 7.0)

0.05% (w/v) NaCl

0.5% (w/v) yeast extract

10 mM $MgSO_4$

2% (w/v) tryptone

2.5 mM KCl

20 mM glucose

Stripping buffer (pH 2.5)

50 mM glycine

Transfer buffer

192 mM glycine

20% (v/v) ethanol

25 mM Tris base

Tris-buffered saline (TBS)

137 mM NaCl

20 mM Tris-HCl, pH 7.6

Tris-buffered saline + Tween 20 (TBST)

0.1% (v/v) Tween 20

137 mM NaCl

20 mM Tris-HCl, pH 7.5

2.1.6 Software

AnalystSoft Inc., VA, USA

StatPlus:mac. Version v6.

Carl Zeiss Ltd, Cambridge, UK

LSM 5 PASCAL software

Li-Cor Biosciences, Lincoln, NE, USA

Image Studio 5.0.21

Molecular Devices, Sunnyvale, CA, USA

MetaMorph 7.5.2

National Institutes of Health, MD, USA

ImageJ 1.46r

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Cell culture growth media for prostate cancer cells

DU145 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin. LNCaP and PC3 cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin. PC3-EmptyVector, PC3-ERK5-17P-Flag and PC3-ERK5-18R-Flag cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 units/mL penicillin, 100 mg/mL streptomycin and 300 μg/mL G418. Cells were maintained in 37 °C humidified cell culture incubator in 5% (v/v) CO_2 with medium replaced every 48 h.

2.2.1.2 Cell culture growth media for mouse embryonic fibroblasts (MEFs)

WT and *AMPK α1*$^{-/-}$ *AMPK α2*$^{-/-}$ KO MEFs were maintained in DMEM supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin. Cells were maintain in 37 °C humidified cell culture incubator in 5% (v/v) CO_2 with medium replaced every 48 h.

2.2.1.3 Passaging of cells

Passaging of cells was carried out when cells reached 80% confluence. Cells in T75 flasks were washed with PBS before being incubated with 2 mL 0.05% (w/v) trypsin in a 37 °C humidified cell culture incubator in 5% (v/v) CO_2. Culture medium was then added to the T75 flask for seeding cells at an appropriate density.

2.2.1.4 Cryopreservation of cell stocks

Cells were washed with PBS before incubation with 2 mL 0.05% (w/v) trypsin in 37 °C humidified cell culture incubator in 5% (v/v) CO_2. Culture medium (4 mL) was added to the flask, and the cell suspension was transferred to a 15 mL vial. The cell suspension was then centrifuged at 350 × g for 5 min and the supernatant was aspirated. The cell pellet was then resuspended in 1 mL freezing medium (medium with 50% (v/v) FCS and 10% (v/v) DMSO). The resuspended cells were then transferred to a polycarbonate container and stored overnight at -80 °C before storage in liquid nitrogen.

2.2.1.5 Resurrection of frozen cell stocks from liquid nitrogen

Cryopreserved cell stocks were removed from liquid nitrogen and rapidly thawed in a 37 °C water bath. Cells were then transferred to a T75 flask containing 11 mL appropriate culture medium as described above. Cells were maintained in a 37 °C humidified cell culture incubator in 5% (v/v) CO_2, medium was aspirated and replaced after cell attachment.

2.2.2 Preparation of cell lysates

Cells grown on cell culture plates were preincubated in serum-free medium for 2 h at 37 °C in 5% (v/v) CO_2 before incubation with various reagents. Depending on the experimental setup, dimethyl sulphoxide (DMSO) was added as solvent vehicle control where applicable. The medium was removed, the cells were washed once with cold PBS and lysis buffer was added. The cell extract was scraped off using a cell lifter and transferred into pre-chilled microcentrifuge tubes. The extracts were centrifuged using a bench top centrifuge at 11, 600 × g for 5 min at 4 °C. The supernatants were stored at -80 °C.

2.2.3 Protein assay

Protein concentrations were determined using the method of Bradford (Bradford, 1976). Reference standards were prepared using bovine serum albumin (BSA) duplicates of 1 µg, 2 µg and 4 µg in 100 µL H_2O. Diluted lysates (100 µL) or

reference standards were added to 1mL of Bradford reagent and the absorbance at 595 nm assessed. The mean absorbance was calculated and protein concentration determined by comparison to the calculated mean A_{595}/µg BSA derived from the linear portion of the BSA reference standard curve.

2.2.4 siRNA transfection

2.2.4.1 Nucleofection

Transfection with small interfering RNA (siRNA) targeted to AMPK α1 (Qiagen Hs_PRKAA1_5 or Dharmacon ON-TARGETplus #06, #07) was performed according to the manufacturer's protocol. Briefly, 1×10^6 cells were harvested by trypsinisation and centrifuged (256 × g, 6 min at room temperature) before being resuspended using the appropriate solution provided (Kit V for PC3, Kit L for DU145, Lonza). Non-silencing siRNA or targeting siRNA (500 nM) were then combined with 100 µL of appropriate solution. Pmax GFP plasmid (2 mg) (Lonza) was used as a control to measure the transfection efficiency. The combination of cells was subject to appropriate transfection program using a Nucleofector II (Lonza) machine in the cuvette provided. After transfection, 400 µL of appropriate medium was added to the cuvette and transferred to a 6cm diameter cell culture plate and incubated at 37 °C in 5% (v/v) CO_2. Transfection efficiency was assessed using fluorescent microscopy (Carl-Zeiss) every 24 h for 3 consecutive days.

2.2.4.2 Lipofectamine

Transfection with siRNA targeted to AMPK α1 (Qiagen Hs_PRKAA1_5 or Dharmacon ON-TARGETplus #06, #07) was performed using Lipofectamine RNAiMAX according to the manufacturer's protocol. Briefly, 1.2×10^5 cells were seeded in a 6 cm diameter Corning cell culture plate for 24 h prior to siRNA transfection. Mixture of 250 µL Opti-MEM and 32 pmol siRNA was diluted with Lipofectamine reagent (5 µL Lipofectamine in 250 µL Opti-MEM) and incubated at room temperature for 25 min. Full medium was then added into the siRNA-

reagent complex to make up a total volume of 3 mL and transferred to each 6 cm plate, which gives a final siRNA concentration of 10 nM.

2.2.5 Recombinant adenoviruses

2.2.5.1 AMPK adenovirus

Adenoviruses expressing a dominant negative (DN) AMPK α mutant (Ad.AMPK-DN, full-length AMPK α1 containing a D157A mutation, Myc-tagged) or GFP control (Ad.GFP) have been described previously (Woods *et al.*, 2000) and were gifts from Dr Fabienne Foufelle, Centre Biomédical des Cordeliers, Paris. The propagation, purification, titration and verification of the AMPK adenovirus were carried out by Dr Silvia Bijland and Dr Sarah Mancini (University of Glasgow).

2.2.5.2 Infection of PC3 and DU145 cells with adenoviruses

Cells (4×10^5) were seeded in one well on a 6-well plate with 2 mL appropriate growth medium, and the plate was incubated at 37 °C in 5% (v/v) CO_2 overnight. The plate was washed with serum-free medium, and 500 µL serum-free medium was added to each well before infection (200 IFU/cell for PC3, 100 IFU/cell for DU145) with Ad.AMPK-DN or Ad.GFP. The plate was then incubated for 3 h at 37 °C, 5% (v/v) CO_2. Appropriate culture medium (500 µL) containing 20% (v/v) FBS was then added to each well for 48 h and incubated at 37 °C in 5% (v/v) CO_2 before experiments were performed.

2.2.6 Plasmid DNA transformation and transfection

2.2.6.1 Plasmid DNA transformation

Empty control plasmid pCMV2-EmptyVector-Flag was made in house at the Cancer Research UK Beatson Institute, Glasgow, UK. Constitutively active MEK5 (Ser313 and Thr317 substituted with Asp) plasmid MSCU-MEK5D (mouse) as previously described, was a generous gift from Dr Jiing-Dwan Lee, The Scripps

Research Institute, CA, USA (Mehta *et al.*, 2003, McCracken *et al.*, 2008). The constitutively active MEK5 (Ser313 and Thr317 substituted with Asp) pCMV-MEK5DD-HA (rat) (C terminal triple human influenza haemagglutinin (HA) tag) was a kind gift from Dr Ruth Cosgrove, Babraham Institute, Cambridge, UK. Briefly, 100 µL of XL-1 Blue competent *E. Coli* cells were thawed on ice before 1 µL plasmid DNA was added for each transformation. The cells were incubated on ice for 15 min before heat shock for 45 sec at 42 °C. 400 µL super optimal broth with SOC medium was added for each transformation, the mixture was then incubated at 37 °C for 1 h. Cells (100 µL) of cells were then spread on a 10 cm diameter 2YT Agar plate with 100 µg/mL Ampicillin and incubated overnight at 37 °C.

2.2.6.2 DNA preparations from *E. coli* (Maxiprep)

A single colony was picked and incubated in 5 mL LB medium overnight at 37 °C. Glycerol stocks were prepared by mixing 625 µL of the resultant *E. Coli* with 375 µL 80% (v/v) glycerol (final concentration 30%) and stored at -80 °C. DNA extraction was undertaken using a Plasmid Maxi Kit (Qiagen) according to the manufacturer's protocol. DNA concentration was determined using a NanoDrop 3300 Fluorosepectrometer (Thermo Scientific).

2.2.6.3 Transfection of PC cells with MEK5 plasmid

Transfection of PC cells was performed using Nucleofection (Lonza) according to the manufacturer's protocol. Briefly, 1×10^6 cells were harvested by trypsinisation and centrifuged (256 × g, 5 min, room temperature) before being resuspended using the Nucleofection Kit V solution. Plasmid (3 µg) was then combined with 100 µL of Kit V solution at room temperature. Cells were subjected to transfection program (T-013) using a Nucleofector II machine in the cuvette provided. After transfection, 400 µL of full medium was added to the cuvette and transferred to a 6 cm diameter Corning cell culture plate and incubated under normal conditions (37 °C, 5% (v/v) CO_2) for 72 h. Transfection efficiency using Nucleofection was analysed using pmaxGFP Vector by fluorescence microscopy.

2.2.7 Immunoblotting

2.2.7.1 SDS-Polyacrylamide Gel Electrophoresis

Samples were prepared as described in Chapter 2.2.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 1.5 mm thick vertical slab gels containing 10% acrylamide. The gels were prepared using Bio-Rad mini-Protean III gel units. The stacking gel consisted of 5% (v/v) acrylamide/0.136% (v/v) bisacrylamide in 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 0.1% (w/v) ammonium peroxidisulphate (APS) and 0.05% (v/v) tetramethylethylenediamine (TEMED). Cell lysates were prepared as described in Chapter 2.2.2. Equal amount of protein were added to 4 × sample buffer and the mixture was heated to 95 °C for 5 min before loading. Prestained broad range protein markers were used as a standard. Gels were electrophoresed using the Bio-Rad Protean III system at a constant voltage of 80 V for stacking and 150 V through the resolving gel. Gels were electrophoresed until the dye front had migrated to the bottom of the gel and good separation of the molecular weight markers had been achieved.

2.2.7.2 Electrophoretic transfer of protein

The gel was removed after electrophoresis and placed on a filter paper pre-wetted with transfer buffer. A pre-wetted sheet of nitrocellulose was placed on top of the gel with a pre-wetted filter paper on top of that. Two sponges were added to either side of "sandwich" which was inserted into the gel holder cassette and transfer was performed using a Bio-Rad mini Protean III trans-blot electrophoretic transfer cell at a constant current of 60 V for 2 h 15 min. The nitrocellulose membrane was then removed from the cassette and stained with Ponceau S to determine the transfer efficiency and protein loading.

2.2.7.3 Probing with primary antibodies

Nitrocellulose membranes were blocked with 5% (w/v) milk in TBS for 30 min at room temperature with gentle shaking. The membrane was washed with TBST (2

× 5 min). Appropriate primary antibody was applied to the membrane in TBST/50% (v/v) Odyssey blocking buffer and incubated overnight at 4 °C with gentle shaking.

2.2.7.4 Probing with secondary antibodies

After overnight incubation with primary antibody, the membrane was then washed with TBST (2 × 5 min) and incubated for 1 h with appropriate fluorescence-labelled secondary antibody in TBST/50% (v/v) Odyssey blocking buffer at room temperature. The membrane was washed with TBST (2 × 5 min) and then TBS (1 × 5 min).

2.2.7.5 Stripping of antibodies from nitrocellulose membranes

Membranes were incubated in stripping buffer for 10 min at room temperature with gentle shaking. The membrane was then washed with TBST (2 × 10 min, 1 × 5 min) Prior to blocking and probing as described in sections 2.2.7.3 and 2.2.7.4.

2.2.7.6 Densitometric analysis of molecular weight species

Visualisation of protein was performed wet using the Li-Cor Sa image system according to the manufacturer's protocols. Quantification of immunoblots was performed using Image Studio software (Li-Cor) and/or Image J software (National Institutes of Health).

2.2.8 WST-1 viability assay

Cell viability assay was performed using the WST-1 (Roche) reagent (tetrazolium salt) in PC3 and DU145 cells according to the manufacturer's protocol based on the principle previously described, which provides estimation of cell viability by measuring metabolic activity (Ishiyama *et al.*, 1993). Briefly, 1×10^4 cells/well were seeded in a 96-well plate using 100 μL/well of growth medium for 24 h allowing for attachment. The cells were washed once with serum-free medium and were subjected to incubation for 2 h in serum-free medium (100 μL/well). Experiments were carried out by adding 100 μL/well serum-free medium

containing appropriate treatment. WST-1 (20 μL/well) was added to each well after 72 h incubation at 37 °C in 5% (v/v) CO_2 and absorption at 492 nm measured with a reference wavelength at 595 nm using a spectrophotometer at 30 min, 60 min, 120 min, 180 min and 240 min. A blank reading was taken using serum-free medium in the absence of cells for normalisation purposes.

2.2.9 BrdU proliferation assay

Cell proliferation assays for PC3, DU145, WT and *AMPK α1*$^{-/-}$ *AMPK α2*$^{-/-}$ KO MEFs were performed using the BrdU (bromodeoxyuridine) cell proliferation assay kit (Millipore) according to the manufacturer's protocol based on the principle previously described, which directly measures DNA synthesis (Magaud *et al.*, 1988). Briefly, 1 × 10^4 cells/well were seeded in a 96-well plate. For PC3 and DU145, cells were seeded using 100 μL/well of growth medium for 24 h allowing for attachment. The cells were washed once with serum-free medium and were subjected to incubation for 2 h in serum-free medium (100 μL/well). Experiments were carried out by adding 100 μL/well serum-free medium containing appropriate treatment. BrdU reagent (20 μL/well) was added to each well after 72 h and incubated for a further 2 h. For WT and *AMPK α1*$^{-/-}$ *AMPK α2*$^{-/-}$ KO MEFs, cells were seeded using 100 μL/well of medium containing 1% (v/v) FBS. Experiments were carried out by adding 100 μL/well 1% (v/v) FBS medium containing appropriate treatment. BrdU reagent (20 μL/well) was added to each well after 24 h and incubated for a further 8 h. All cells were incubated at 37 °C in 5% (v/v) CO_2. The plate was then fixed and developed for colourimetric analysis according to the protocol. Absorption at 492 nm was measured with a reference wavelength at 595 nm using SPECTROstar Nano microplate reader (BMG Labtech). A blank reading was taken using medium only in the absence of cells for normalisation purposes.

2.2.10 Apoptosis signalling array

The apoptosis signalling array was performed using the PathScan Stress and Apoptosis Signaling Antibody Array Kit (Cell Signaling Technology) in PC3 cells.

Briefly, PC3 cells were seeded in 10 cm diameter cell culture dishes until 90% confluent at 37 °C in 5% (v/v) CO_2. The cells were then washed using serum-free culture medium, and incubated under serum-free conditions for 2 h in 37 °C in 5% (v/v) CO_2. Reagents were then added and incubated for different periods of time up to 72 h according to the experimental design. The dishes were then washed with ice-cold PBS before lysis with 300 μL/dish ice-cold lysis buffer (Cell Signalling Technology) supplemented with protease inhibitor cocktail (Roche). Protein lysates were transferred to microcentrifuge tubes and incubated on ice for 10 min prior to centrifugation (11, 600 × g, 4 °C, 5 min). Supernatants were stored at -20 °C before use and protein assays were performed as described before. Equal amount of protein (50 μg) was loaded to each well after dilution with array diluent buffer. The assay kit was incubated overnight at 4 °C with gentle shaking. The array was detected as per the protocol and image was captured dry using the Li-Cor Sa image system. Quantification of the array was performed using Image Studio software (Li-Cor).

2.2.11 Monolayer wound healing assay

The monolayer wound healing assay for PC3 and DU145 cells was performed as described previously (Wang *et al.*, 2009). Briefly, 3×10^5 cells/well were seeded in a 6-well plate using 3.5 mL of appropriate growth medium and incubated at 37 °C in 5% (v/v) CO_2 for 72 h until fully confluent. Cells were then subjected to incubation for 2 h in serum-free medium before being scratched using a sterile 20-200 μL pipette tip. Cells were then treated with appropriate reagents depending on the experimental design. Still photographs were taken continuously every 15 min over a 22 h period using TE2000 time-lapse microscope (Nikon) at 10 × magnification. Image J software was used to measure wound size, and three different fields were analysed in each experiment setup, and four cells were tracked on each edge.

2.2.12 Transwell migration assay

Migration assay for PC3 and DU145 cells was performed in 24-well plates across 8μm pore cell culture inserts as previously described (Choudhury *et al.*, 2014). Briefly, 5×10^4 cells were suspended in serum-free medium in the insert. Serum-free medium or medium containing 10% (v/v) FBS was added in the well. Different compounds were added to both the inserts and the wells as per the experimental setup, the cells were incubated over a period of 21 h allowing for migration. The migrated cells in the insert were then fixed using methanol for 30 min at -20 °C and then stained with haematoxylin for 30 min at room temperature. The insert was washed with dH_2O. The membrane with the migrated cells was mounted onto slides using DPX mountant. The slides were analysed under BM50 microscope (Olympus) at 10 × magnification, four different fields were analysed in each experiment setup.

2.2.13 Immunofluorescent labelling of cells

PC3 and DU145 cells were seeded in 12-well plates (1.7×10^5 cells/well) containing sterile glass coverslips and incubated overnight allowing for attachment at 37 °C in 5% (v/v) CO_2. The plate was then infected with adenoviruses as described above in 2.2.5. After 48 h, the medium was aspirated, and the plate was washed once with warm PBS. Coverslips were fixed with 3% (w/v) paraformaldehyde at room temperature for 20 min, washed twice with 20 mM glycine/PBS for quenching, and then twice with PBS. The coverslips were then incubated in permeabilisation buffer (0.1% (v/v) Triton X-100 in PBS) for 4 min and washed with PBS three times. The coverslips were then blocked in immunofluorescence (IF) buffer. Coverslips were subsequently incubated in primary antibody and 1:20 Texas Red-X Phalloidin (Thermo Fisher Scientific) in IF buffer for 1 h at room temperature, and washed three times with IF buffer afterwards. Coverlips were then incubated in secondary antibodies for 1 h at room temperature in the dark, and washed three times with IF buffer afterwards. 1:200 Red Dot (Biotium) was used for nuclear staining. The coverslips were then mounted onto slides using a drop of Immunomount (Thermo Scientific) and stored in dark.

2.2.14 Confocal microscopy

Mounted coverslips were analysed using an oil immersion objective (63 × magnification) on a Zeiss Axiovert 200M confocal microscope (Carl Zeiss) equipped with LSM5 PASCAL Exciter instrument (50% output). An argon laser was used to excite 488 nm Fluor Dyes and GFP fusion proteins. The helium neon laser was used to excite 633 nm Red Dot. The helium neon laser was used to excite 543 nm Texas Red-phalloidin. Zeiss Pascal software was used to collect images (10 fields per slide).

2.2.15 Immunohistochemical analysis using tissue microarray

Formalin-fixed and paraffin-embedded tissue microarray (TMA) samples were analysed using immunohistochemistry by Dr Yashmin Choudhury and Dr Imran Ahmad (University of Glasgow) as described (Choudhury *et al.*, 2014). Briefly, TMA sections were incubated with relevant primary antibodies at 4 °C overnight, and were further incubated with HRP-labelled secondary antibody at room temperature for 1 h. The cores were scored blindly by two independent researchers and a Histoscore (H-score), the quantification of staining, was generated (Viollet *et al.*, 2010).

2.2.16 Statistics

Data analysis was performed using a Student's t-test (two-tailed) unless stated otherwise. A p value <0.05 is deemed statistically significant in comparison. Results are reported as mean +/- standard error and are from three independent experiments unless otherwise stated.

Chapter 3. Characterisation of AMPK expression and activation in human prostate cancer cell lines

3.1 Introduction

3.1.1 Current understanding of AMPK upstream kinases in prostate cancer cells

LKB1 and CaMKK2 are the two primary upstream kinases that activate AMPK via phosphorylation at Thr172 (Hardie, 2007b, Hardie, 2015). LKB1 is recognised as a tumour suppressor (Hemminki *et al.*, 1998, Bardeesy *et al.*, 2002), and *in vitro* studies have shown that the prostate cancer (PC) cell line DU145 does not express LKB1, yet AMPK can surprisingly be phosphorylated and activated by AICAR in such cells (Yun *et al.*, 2005), despite LKB1 being essential for AICAR mediated AMPK phosphorylation (Hutber *et al.*, 1997). In addition, transgenic animal models have shown that loss of LKB1 can lead to neoplasia in mice (Pearson *et al.*, 2008). CaMKK2, responsible for the Ca^{2+}/calmodulin kinase cascade (Colomer and Means, 2007), is reported to be elevated in PC cell lines especially in response to AR stimulation (Frigo *et al.*, 2011, Massie *et al.*, 2011). It is also suggested that there may be a signalling feedback loop between CaMKK2 and AR (Karacosta *et al.*, 2012). However, the expression/function of and crosstalk between LKB1 and CaMKK2 in the context of the AMPK signalling pathway in PC cell lines is poorly understood.

3.1.2 Expression of AMPK subunits in prostate cancer cells

AMPK has been considered as a therapeutic target in cancer (Sanli *et al.*, 2012a). In PC, however, it is unknown whether any of the AMPK subunits have a significant role in carcinogenesis. The expression of AMPK subunits was not researched in detail before the start of this project. Sanli and colleagues also demonstrated that all tested AMPK subunit isoforms are expressed in human lung, prostate and breast cancer cell lines, and different levels of expression can be seen in human prostate PNT1A, PC3 and 22Rv1 cells (Sanli *et al.*, 2012b).

3.1.3 AMPK activators used in prostate cancer cells in vitro

Metformin, the most common anti-diabetic medication activates AMPK by altering the AMP/ATP or ADP/ATP ratio (Zhou *et al.*, 2001, Hawley *et al.*, 2010, Bijland *et al.*, 2013). Furthermore, metformin has been reported to have an anti-cancer effect in *in vitro* studies using PC cells (Ben Sahra *et al.*, 2008, Ben Sahra *et al.*, 2010a, Malaguarnera *et al.*, 2014). AICAR is phosphorylated to ZMP that mimics AMP to activate AMPK *in vitro* (Lopez *et al.*, 2003, Guigas *et al.*, 2006, Bijland *et al.*, 2013). There are many studies which have used AICAR, A769662 and metformin as AMPK activators both *in vitro* and *in vivo* (Xiang *et al.*, 2004, Goransson *et al.*, 2007, Sanders *et al.*, 2007, Huang *et al.*, 2008, Park *et al.*, 2009, Zhou *et al.*, 2009, Ben Sahra *et al.*, 2010a), yet at the beginning of this study, only AICAR had been used as an AMPK activator in PC3, DU145 and LNCaP cell lines (Xiang *et al.*, 2004, Sauer *et al.*, 2012).

3.1.4 Manipulation of AMPK expression and activity

To date, several tools have been used to manipulate AMPK expression *in vitro*. AMPK siRNA targeting the *PRKAA1* gene has been used to knockdown AMPK α1 levels in LNCaP cells (Chhipa *et al.*, 2011). In addition, adenoviruses expressing either DN or constitutively activated AMPK α1 mutants have been used to study the role of AMPK in multiple cell lines including PC cells (Woods *et al.*, 2000, Sakoda *et al.*, 2002, Xing *et al.*, 2003, Hwang *et al.*, 2008, Canto *et al.*, 2009, Zhou *et al.*, 2009).

3.1.5 Aims

Previous evidence has shown that different AMPK subunits may have different roles in both physiological and pathological conditions (Feng *et al.*, 2007, O'Neill *et al.*, 2011). In addition, at least six mechanisms have been identified for AMPK activation using different activators (Hawley *et al.*, 2010). In fact, different AMPK subunits confer different sensitivity to some activators. For example, in cells that lack LKB1, basal AMPK α2 isoform activity is reduced, and AICAR's ability to stimulate AMPK phosphorylation is reduced (Sakamoto *et al.*, 2005). The

activation of AMPK by A769662, a more potent and specific AMPK activator than AICAR, is selective for AMPK complexes containing the β1 isoform (Sanders *et al.*, 2007, Scott *et al.*, 2008), yet the effects of A769662 on PC cell lines had not been reported prior to the current study.

PC3, DU145 and LNCaP are the most well-characterised PC cell lines (Sobel and Sadar, 2005). PC3 and DU145 cell lines are androgen independent, whereas LNCaP is androgen sensitive (Sobel and Sadar, 2005). DU145 cells do not express the AMPK kinase LKB1 (Yun *et al.*, 2005, Yun *et al.*, 2009). The PC3M cell line is derived from the PC3 cell line, and exhibits a more aggressive biological behaviour (Kozlowski *et al.*, 1984). The LNCaP-AI cell line is androgen-independent and derived from androgen-dependent LNCaP cells (Lu *et al.*, 1999). Unlike the androgen-independent PC3 cells, AR is still expressed in LNCaP-AI cells (Lu *et al.*, 1999). CWR22 cells are derived from mice xenograft, which is known for its high expression of PSA and the epidermal growth factor receptor (EGFR) (Wainstein *et al.*, 1994). Cell lines bearing these different molecular properties were therefore used to examine the expression of AMPK subunit isoforms and responses to activators (A769662 and AICAR) that stimulate AMPK by different mechanisms. (Table 3.1)

Cell Lines	Origin	Androgen receptor	LKB1	CaMKK2
CWR22	Mice xenograft	+	+	+
DU145	Brain metastasis	-	-	+
PC3	Bone metastasis	-	+	+
PC3M	From PC3	-	+	+
LNCaP	Lymph node metastasis	+	+	+
LNCaP-AI	From LNCaP	-	+	?

Table 3.1 The origin and molecular profiles of prostate cancer cells

CWR22 cells were derived from a mice xenograft established from osseous metastasis. DU145 cells were derived from brain metastatic cells. PC3 cells were derived from bone metastatic cells PC3M cell line was established from a PC3 xenograft and exhibits more aggressive invasiveness than PC3 cells. LNCaP cells were from derived from lymph node metastatic cells. The LNCaP-AI cell line was selected from a fast growing clone of LNCaP cells, which were androgen independent. Each cell line has differential morphology and molecular properties.

3.2 Results

3.2.1 Expression of AMPK upstream kinases in prostate cancer cell lines

Using a panel of human PC cell lines maintained in full serum supplement culture conditions, the baseline levels of AMPK α, phospho-AMPK Thr172, ACC and phospho-ACC Ser79 as well as the two recognised AMPK upstream kinases, LKB1 and CaMKK2 were assessed. In addition, baseline phospho-Akt Ser473 and Akt levels were also assessed. Baseline phosphorylation of the AMPK substrate, phospho-ACC Ser79 and phospho-AMPK Thr172 varied among the cell lines, with higher phospho-ACC Ser79 observed in CWR22 and PC3M cells. DU145 cells were the only cells to lack LKB1, and CaMKK2 was detected in all cell lines, although multiple species were observed at around the predicted molecular mass. The level of phospho-Akt Ser473 varied among the cell lines, and being highest in LNCaP and LNCaP-AI cells. (Figure 3.1) PC3, DU145 and LNCaP cells were used to carry out further experiments as this combination includes cells deficient in different signalling pathway intermediates including PTEN and LKB1. Based on previous experience, all experiments were conducted in serum-free conditions to minimise the interference of growth/hormone presented in serum as well as reducing the level of the albumin signal which was apparent in preliminary experiments under conditions where serum was present (data not shown).

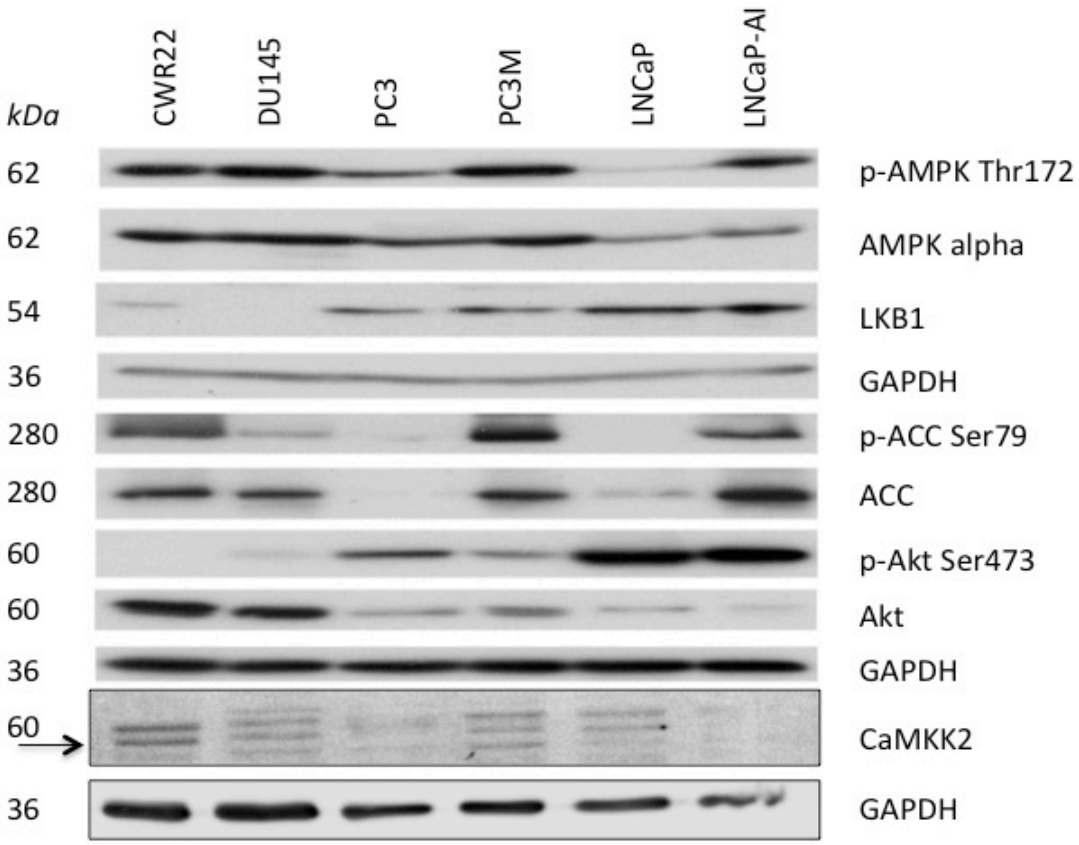


Figure 3.1 Baseline AMPK and AMPK upstream kinase protein levels and phosphorylation in prostate cancer cell lines

Prostate cancer cell lines (CWR22, DU145, PC3, PC3M, LNCaP and LNCaP-AI) were incubated for 2 h in serum-free medium and lysates prepared. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. Representative immunoblots are shown, repeated on at least three occasions. DU145 cells lacked LKB1. Highest phospho-ACC Ser79 levels were observed in CWR22 and PC3M cells. Phospho-Akt Ser473 levels were highest in LNCaP and LNCaP-AI cells. Multiple species were detected due to the quality of CaMKK2 antibody, with the predicted molecular mass of CaMKK2 indicated by the arrow. GAPDH was used as loading control.

The top nine immunoblots were performed by Dr Yashmin Choudhury (University of Glasgow) and are reproduced under the Creative Commons Attribution License using Figure 1A from article "AMP-activated protein kinase (AMPK) as a potential therapeutic target independent of PI3K/Akt signaling in prostate cancer" by Choudhury et al, Oncoscience (2014); 1(6) 446-456.

3.2.2 AMPK subunits expression in prostate cancer cell lines

In order to examine the expression of AMPK subunit isoforms in each PC cell line, lysates from three PC cell lines (PC3, DU145, LNCaP) were assessed by immunoblotting with isoform-specific antibodies. Both alpha isoforms are present in PC3, DU145 and LNCaP cells. Although there is species corresponding to β1, there is no β2 species observed in DU145 cells. It is difficult to interpret the levels of gamma isoforms since the antibodies are not highly specific, although levels of γ1 and γ3 are lower in DU145 cells. These observations indicate that AMPK subunit isoform protein levels differed in PC cells lines. (Figure 3.2)

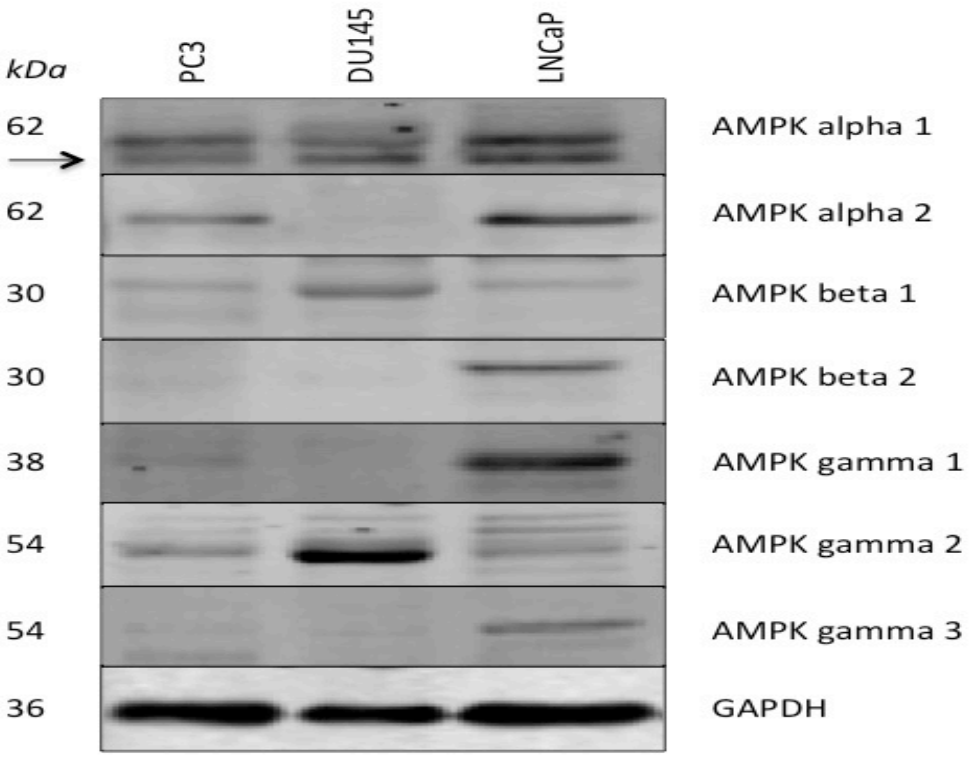


Figure 3.2 AMPK subunit isoform protein levels in prostate cancer cell lines
PC3, DU145 and LNCaP cells were incubated for 2 h in serum-free medium and lysates prepared. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. In DU145 cells, there was no β2 species and levels of γ1 and γ3 are lower. GAPDH was used as loading control. Experiments were repeated at least three times with representative blots shown.

3.2.3 Activation of AMPK in prostate cancer cells

To determine the optimal conditions for AMPK activation in PC cell lines, two AMPK activators were utilised, namely AICAR and A769662. Firstly, seperate time-dependent assays carried out using 1 mM AICAR and 100 μM A769662. A concentration-dependent assay was then followed with AICAR and A769662 at different concentrations. AICAR stimulated phospho-ACC Ser79 in a time- and concentration-dependent manner in all three PC cell lines, with significant stimulation observed at 60 min (LNCaP), 120 min (PC3 and DU145), at 1 mM. A769662 also stimulated phospho-ACC Ser79 in a time- and concentration-dependent manner in all three PC cell lines, with significant stimulation observed at 30 min (DU145), 60 min (PC3 and LNCaP), at 100 μM. AMPK Thr172 phosphorylation was also analysed at the same time. However, changes in the level of the phospho-AMPK Thr172 species observed was not as robust or consistent as with phospho-ACC Ser79 (data not shown). Therefore phospho-ACC Ser79 was used as surrogate to analyse AMPK activation (Figures 3.3 to 3.7).

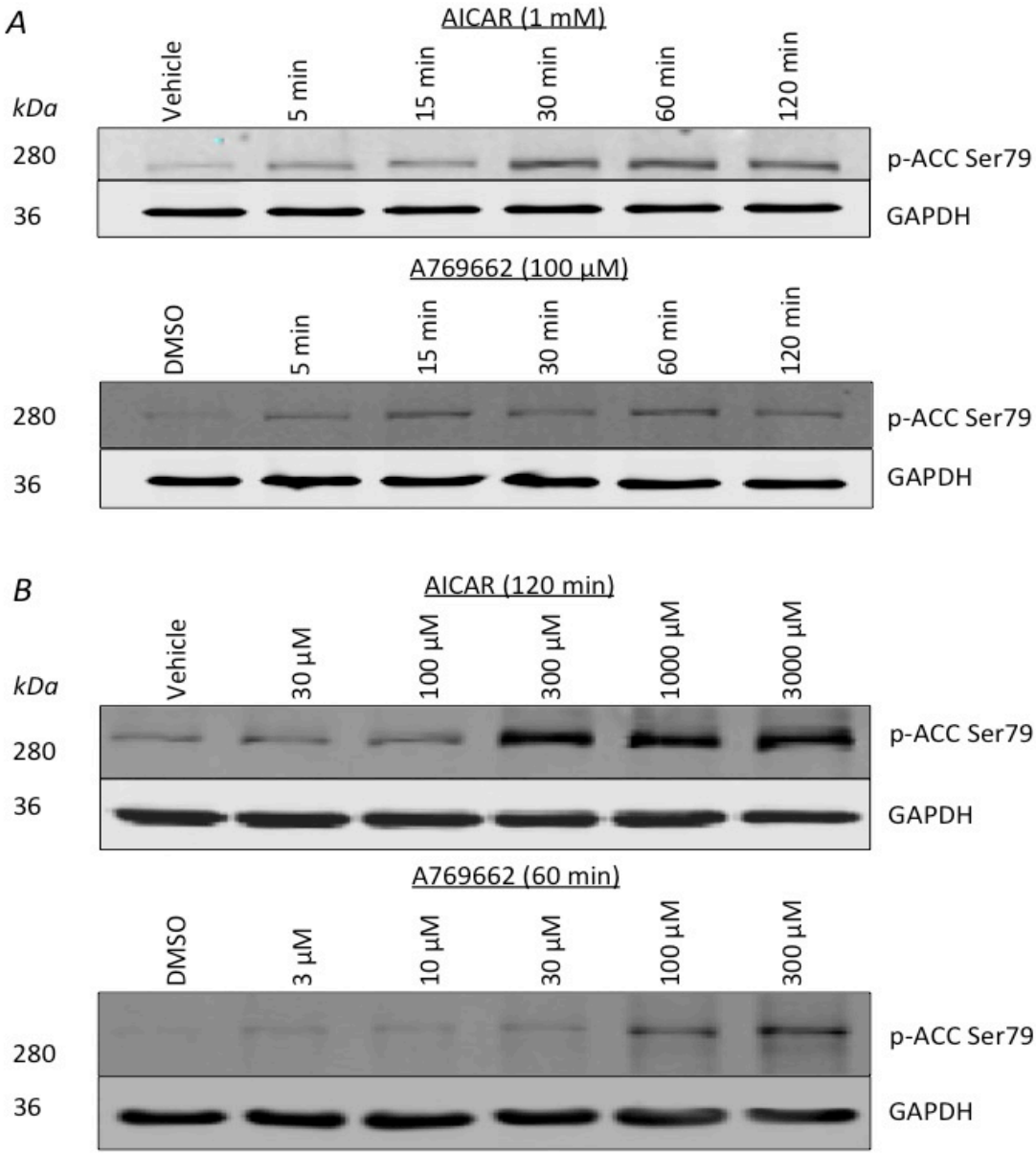


Figure 3.3 AMPK activation by AICAR and A769662 in PC3 cells

In PC3 cells, both AICAR and A769662 activate AMPK (as assessed by phospho-ACC Ser79) in a concentration- and time-dependent manner. Cells were incubated for 2 h in serum-free medium before incubation with the indicated concentrations of AICAR or A769662 for various times and lysates prepared. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots for each time-course (N=3). (B) Representative blots for concentration-dependence (N=3).

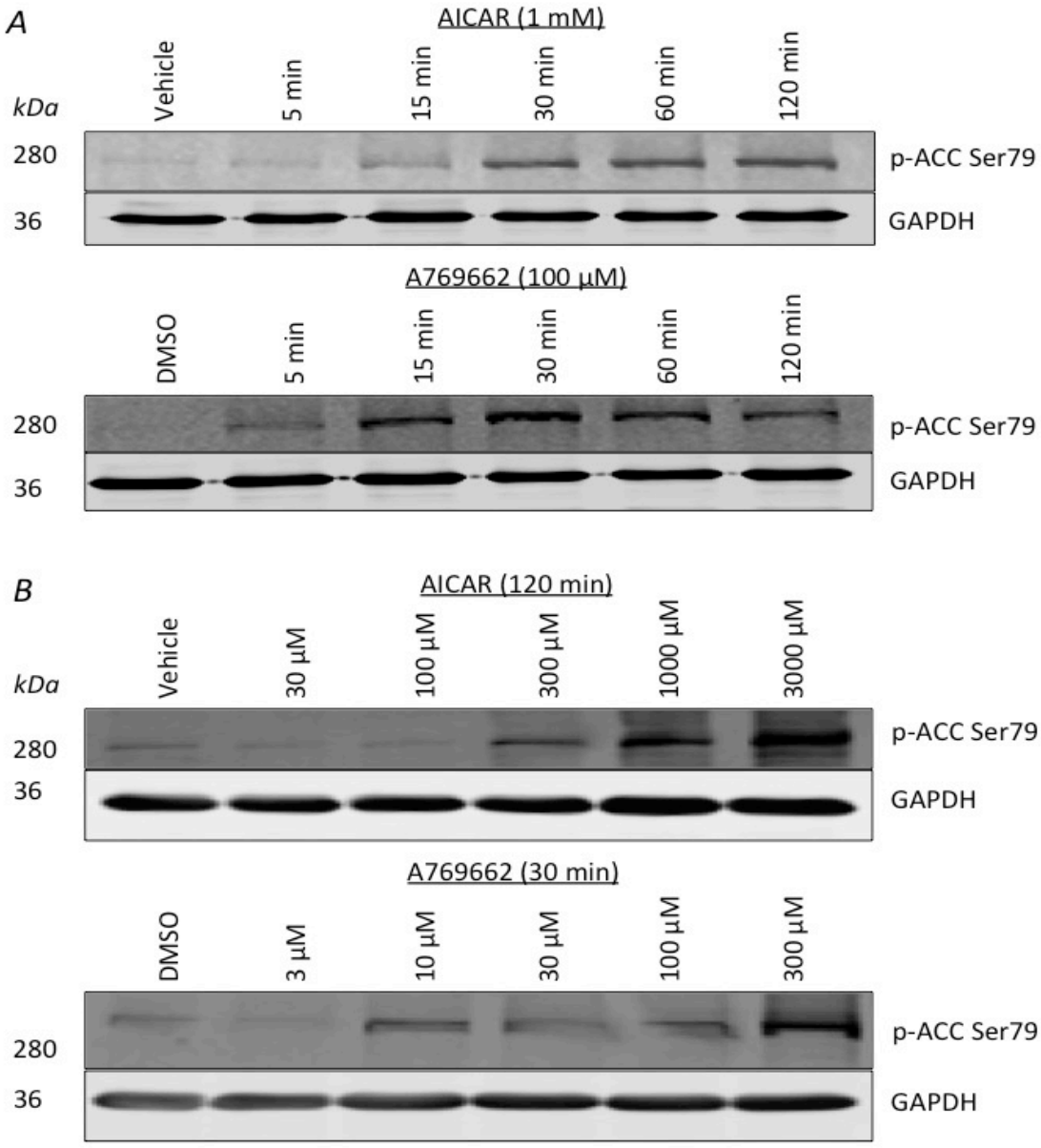


Figure 3.4 AMPK activation by AICAR and A769662 in DU145 cells

In DU145 cells, both AICAR and A769662 activate AMPK (as assessed by phospho-ACC Ser79) in a concentration- and time-dependent manner. Cells were incubated for 2 h in serum-free medium before incubation with AICAR or A769662 for various times and at various concentrations and lysates prepared. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots for each time-course (N=3). (B) Representative blots for concentration-dependence (N=3).

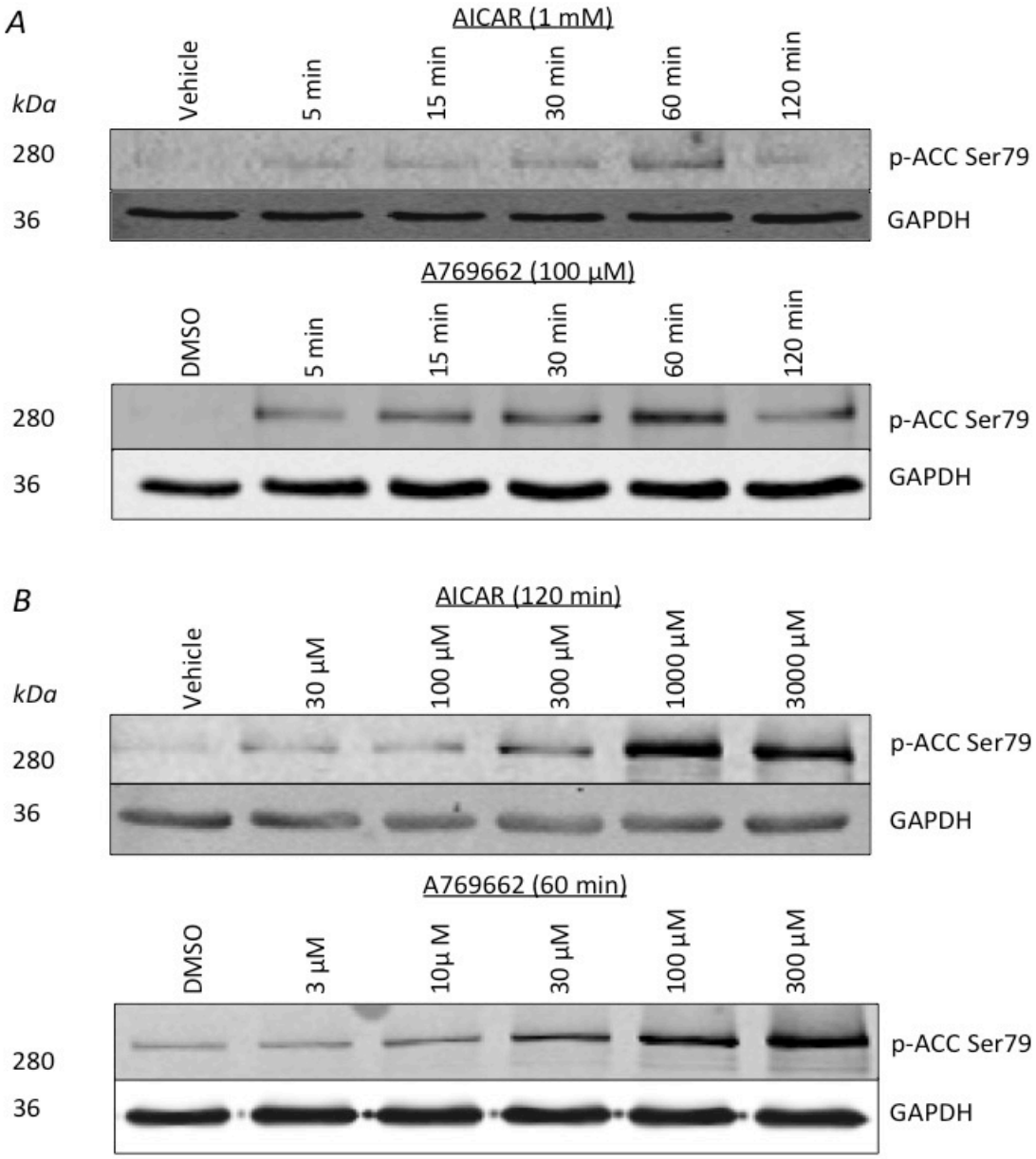


Figure 3.5 AMPK activation by AICAR and A769662 in LNCaP cells

In LNCaP cells, both AICAR and A769662 activate AMPK (as assessed by phospho-ACC Ser79) in a concentration- and time-dependent manner. Cells were incubated for 2 h in serum-free medium before incubation with AICAR or A769662 for various times and at various concentrations and lysates prepared. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots for each time-course (N=3). (B) Representative blots for concentration-dependence (N=3).

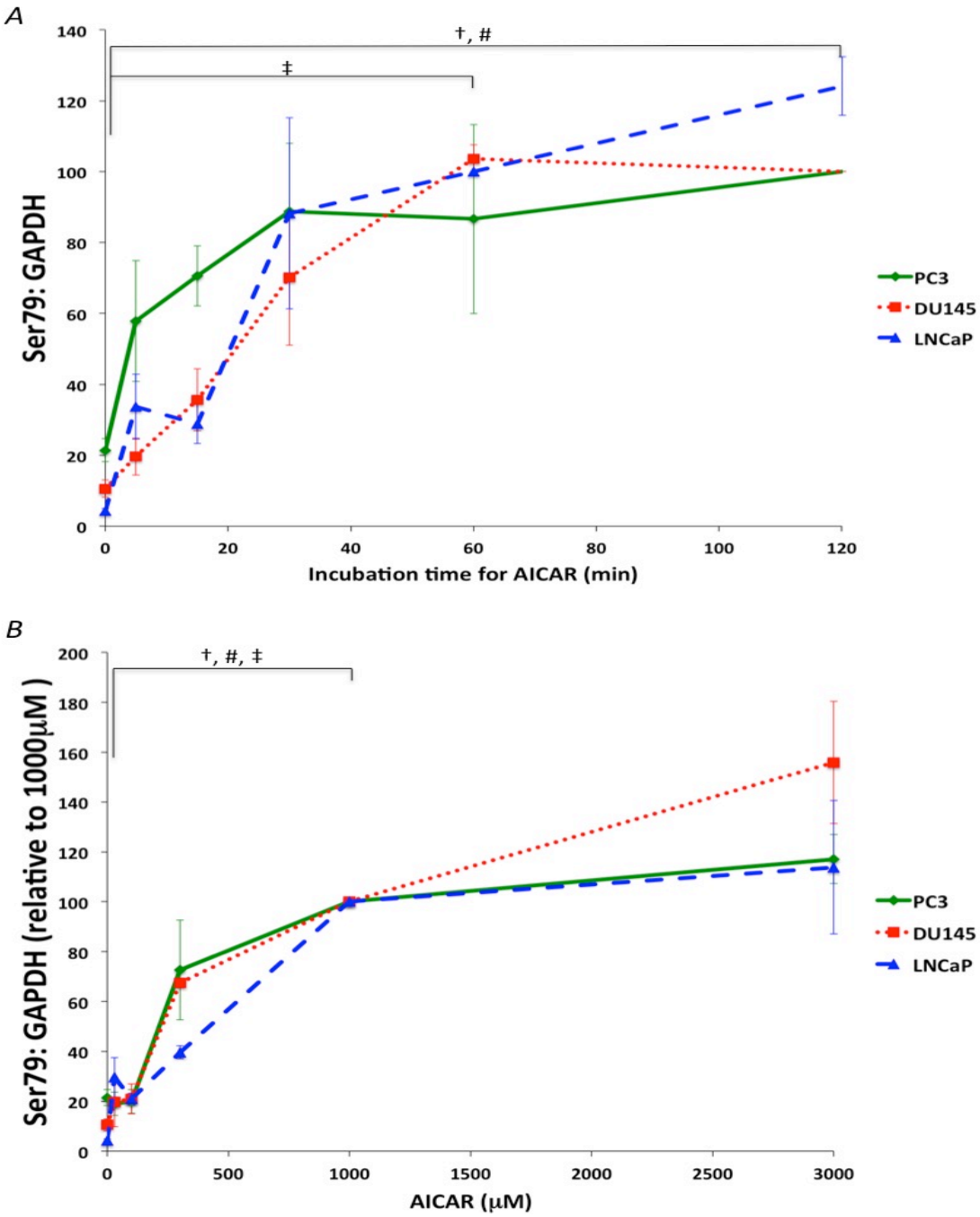


Figure 3.6 AMPK activation by AICAR in prostate cancer cell lines

Quantification of phospho-ACC Ser79 relative to GAPDH in Figures 3.5, 3.6 and 3.7. (A) Time-course response after AICAR incubation (1 mM), PC3 and DU145 were normalised to 120 min, LNCaP were normalised to 60 min ($p<0.01$, †: PC3, N=5, #: DU145, N=5, ‡: LNCaP, N=6). (B) Concentration-dependent curve of AICAR incubation at 120 min, normalised to 1 mM ($p<0.01$, †: PC3, N=5, #: DU145, N=5, ‡: LNCaP, N=6).

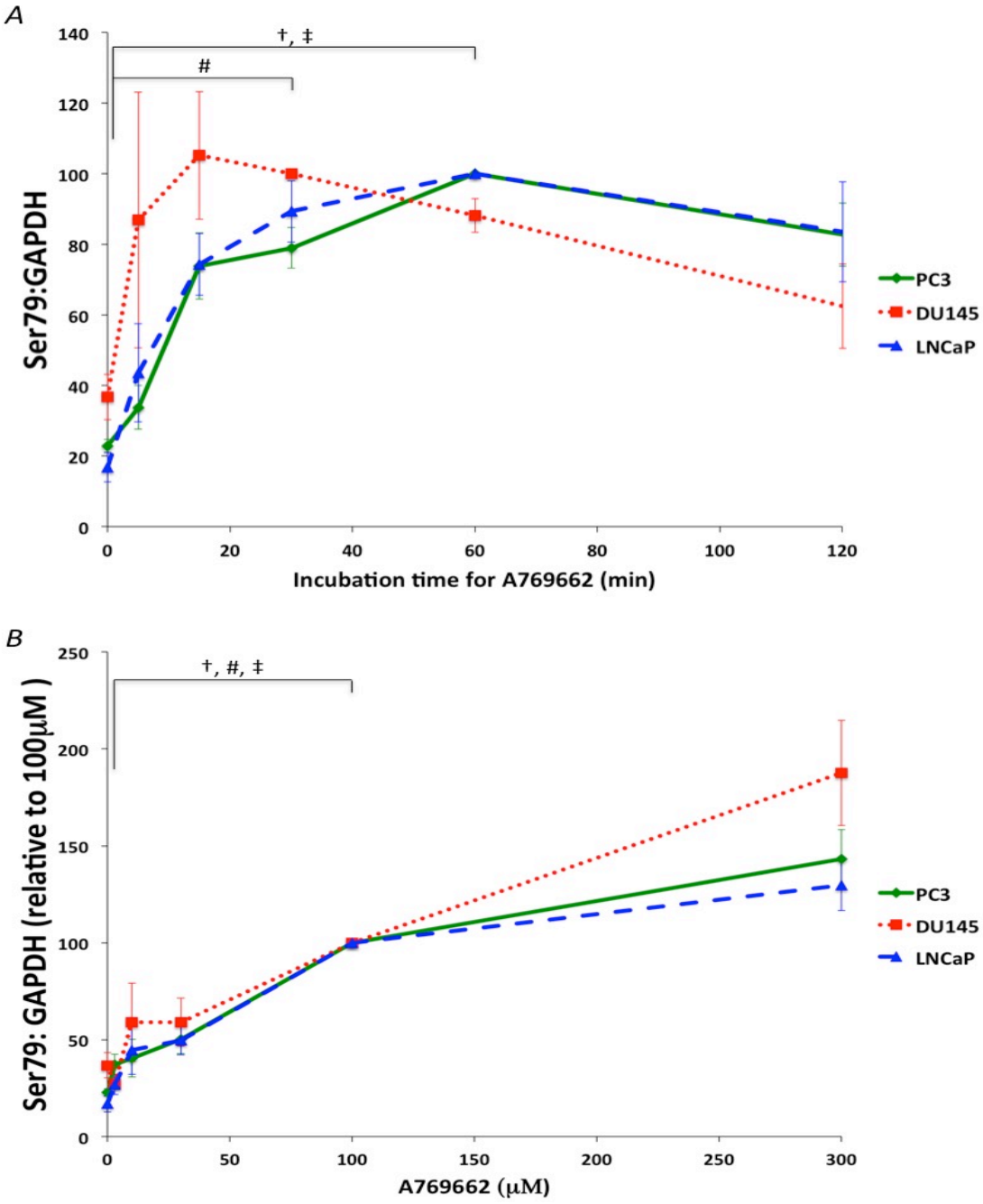


Figure 3.7 AMPK activation by A769662 in prostate cancer cell lines

Quantification of phospho-ACC Ser79 relative to GAPDH in Figures 3.5, 3.6 and 3.7. (A) Time-course response after A769662 incubation (100 μM) PC3 and LNCaP were normalised to 60 min, DU145 were normalised to 30 min ($p<0.01$, †: PC3, N=5, #: DU145, N=5, ‡: LNCaP, N=6). (B) Concentration-dependent curve of A769662 incubation at 60 min (PC3 and LNCaP) and 30 min (DU145) ($p<0.01$, †: PC3, N=5, #: DU145, N=5, ‡: LNCaP, N=6).

3.2.4 AMPK siRNA knockdown in androgen-independent prostate cancer cells

To determine whether any of the observed effects of A769662 or AICAR are AMPK-dependent, a method to specifically down-regulate AMPK activity was required. Different technical approaches were used in combination with various targeting sequences in order to establish a better knockdown. Initially, nucleofection of siRNA was assessed, yielding a transfection efficiency of approximately 50% after 72 h as measured by the pmaxGFP plasmid in both PC3 and DU145 cell lines (data not shown). Furthermore, nucleofection with ON-TARGETplus siRNA targeted against AMPK α1 was similarly of a low efficiency (data not shown) and despite efforts to optimise the nucleofection protocol, satisfactory knockdown of AMPK α1 was not achieved.

Lipofectamine-mediated transfection of PC3 and DU145 cells with siRNA targeting the *PRKAA1* gene resulted in a reduction in AMPK α expression after 72 h by 80% and 40% in each cell line respectively (Figures 3.8 and 3.9). Despite the marked down-regulation of AMPK α levels in PC3 cells, basal phospho-ACC Ser79 was only reduced by 40% - 50% in both cell lines (Figures 3.8 and 3.9). Furthermore, AICAR- and A769662-stimulated AMPK activity, as assessed by phospho-ACC Ser79, was not markedly affected by lipofectamine-mediated down-regulation of AMPK with targeted siRNA. Therefore, an alternative approach other than siRNA for down-regulation of AMPK activity is warranted. The AICAR and A769662-stimulated experiments were carried out in the presence or absence of epidermal growth factor (EGF), as EGF was used as the stimulus of the mitogen-activated protein kinase (MAPK) and PI3K/Akt signalling pathways in later chapters. It is clear that EGF had no effect on siRNA transfection. (Figures 3.10 and 3.11)

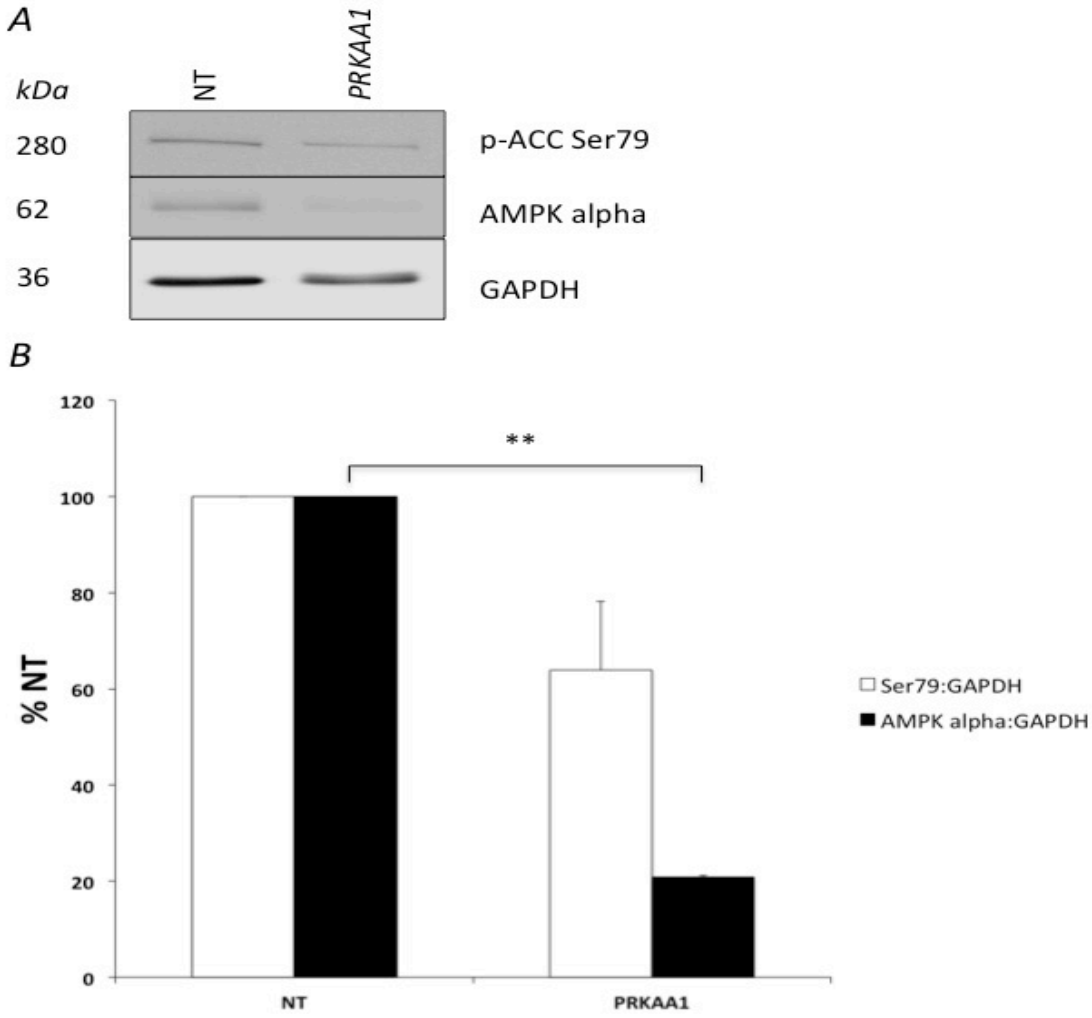


Figure 3.8 siRNA-mediated down regulation of AMPK in PC3 cells

PC3 cells were seeded 24 h before siRNA transfection. *PRKAA1* and non-coding (NT) control siRNA were transfected using Lipofectamine RNAiMAX for 72 h at a concentration of 10 nM. Cells were incubated for 2 h in serum-free medium and lysates prepared. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of AMPK levels and ACC phosphorylation level (**: $p<0.01$, N=3).

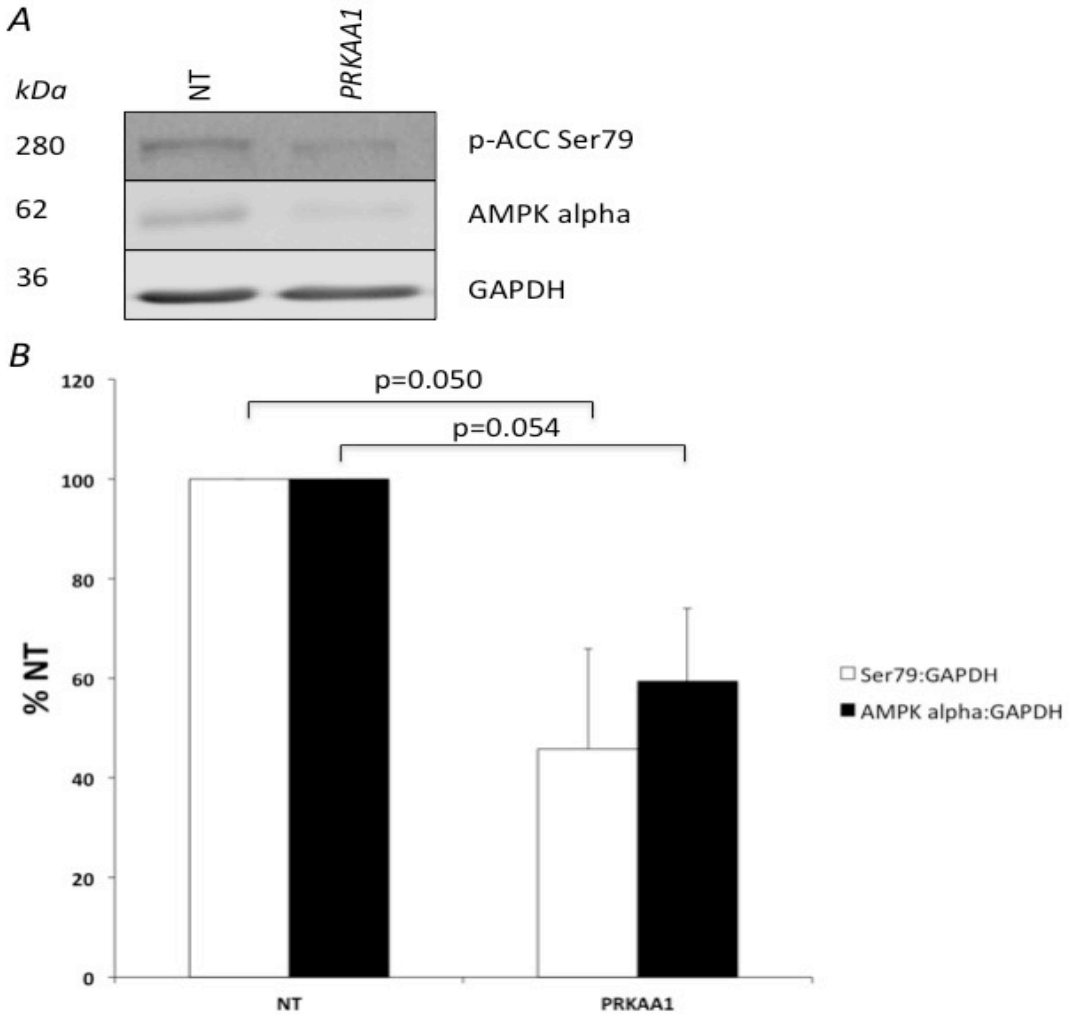


Figure 3.9 siRNA-mediated down regulation of AMPK in DU145 cells

DU145 cells were seeded 24 h before siRNA transfection. *PRKAA1* and non-coding (NT) control siRNA were transfected using Lipofectamine RNAiMAX for 72 h at a concentration of 10 nM. Cells were incubated for 2 h in serum-free medium and lysates prepared. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of AMPK levels and ACC phosphorylation level (N=3).

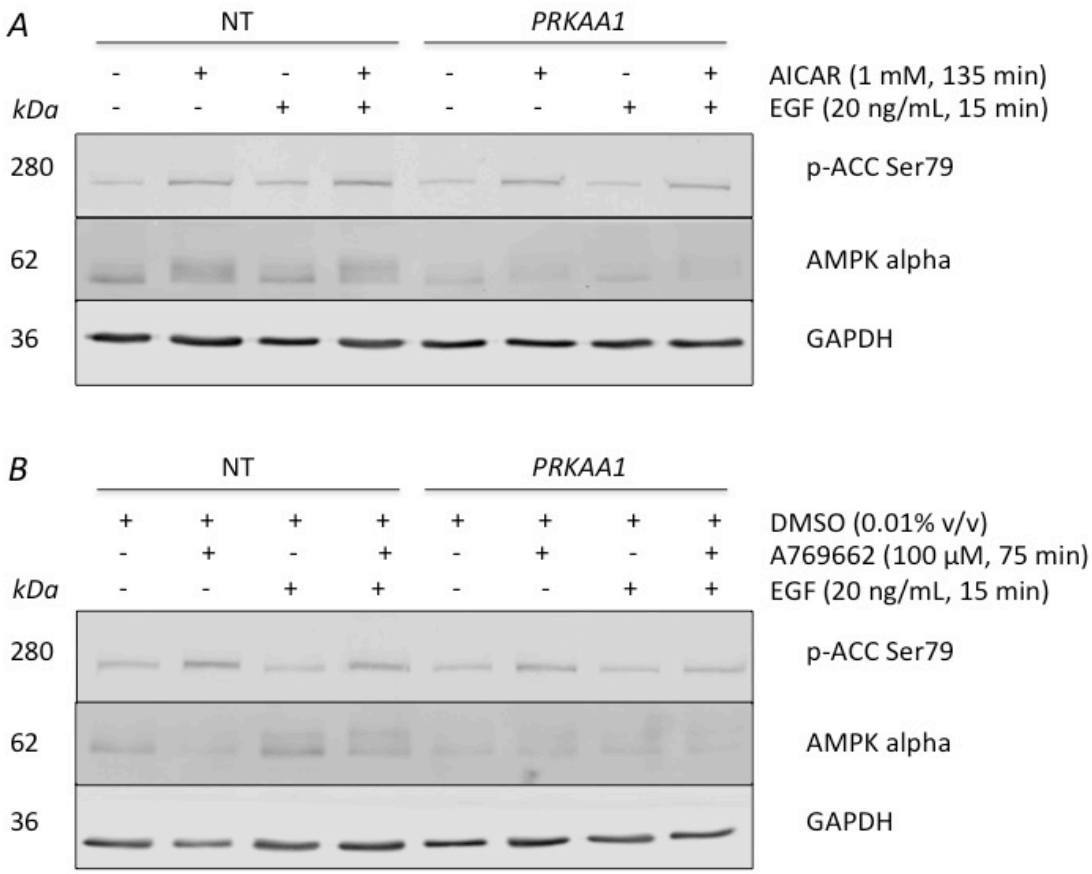


Figure 3.10 Effect of siRNA-mediated down-regulation of AMPK on AICAR and A769662-stimulated ACC phosphorylation in PC3 cells

PC3 cells were seeded 24 h before siRNA transfection. *PRKAA1* and non-coding (NT) control siRNA were transfected using Lipofectamine RNAiMAX for 72 h at a concentration of 10 nM. Cells were then incubated for 2 h in serum-free medium before incubation in the presence or absence of (A) AICAR or (B) A769662. EGF (20 ng/mL) was added 15 min prior to lysates preparation. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with antibodies indicated. GAPDH was used as loading control. Representative blots are shown (N=3).

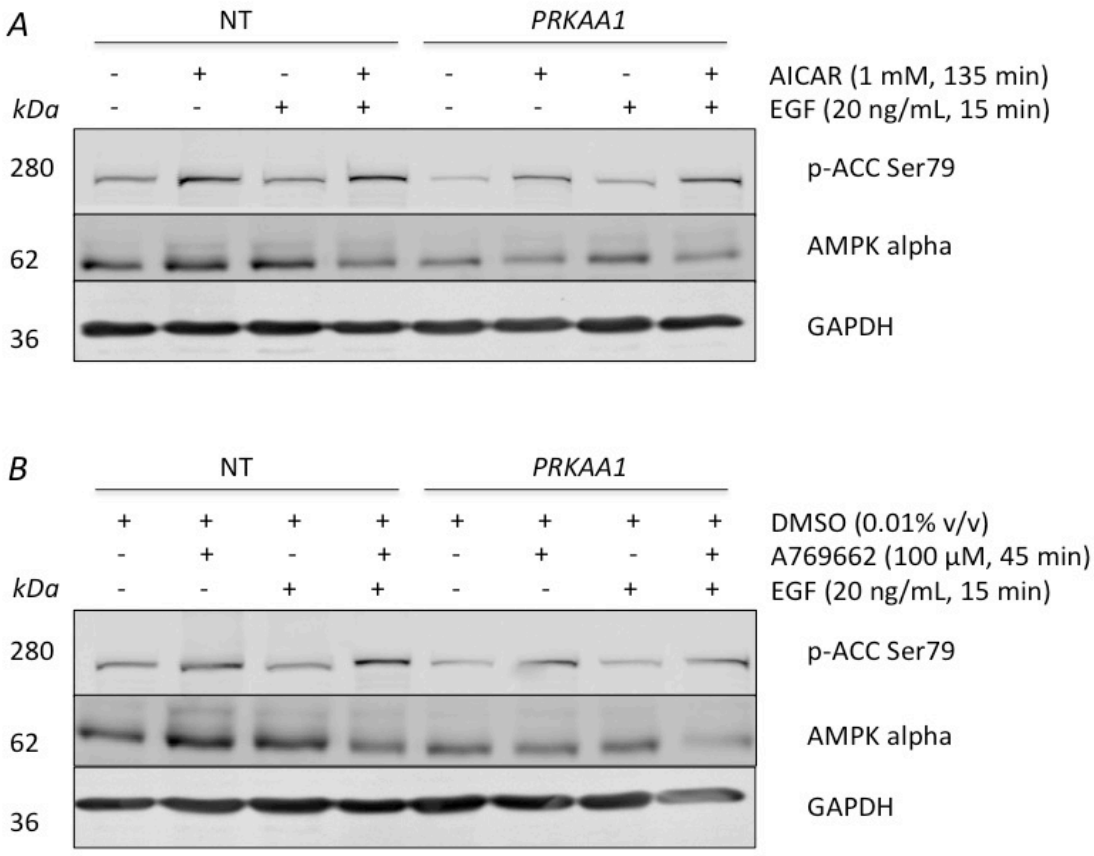


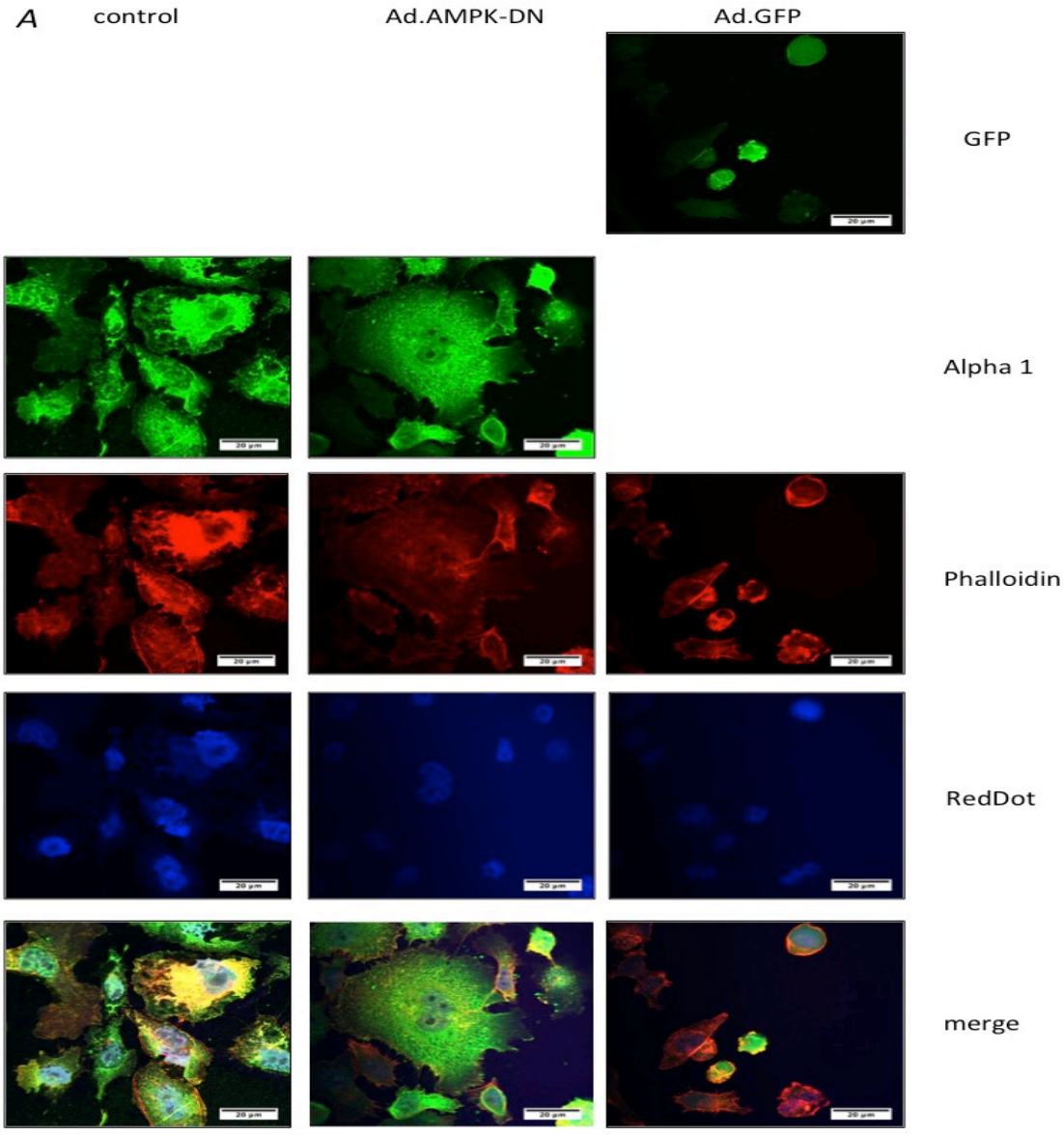
Figure 3.11 Effect of siRNA-mediated down-regulation of AMPK on AICAR and A769662-stimulated ACC phosphorylation in DU145 cells

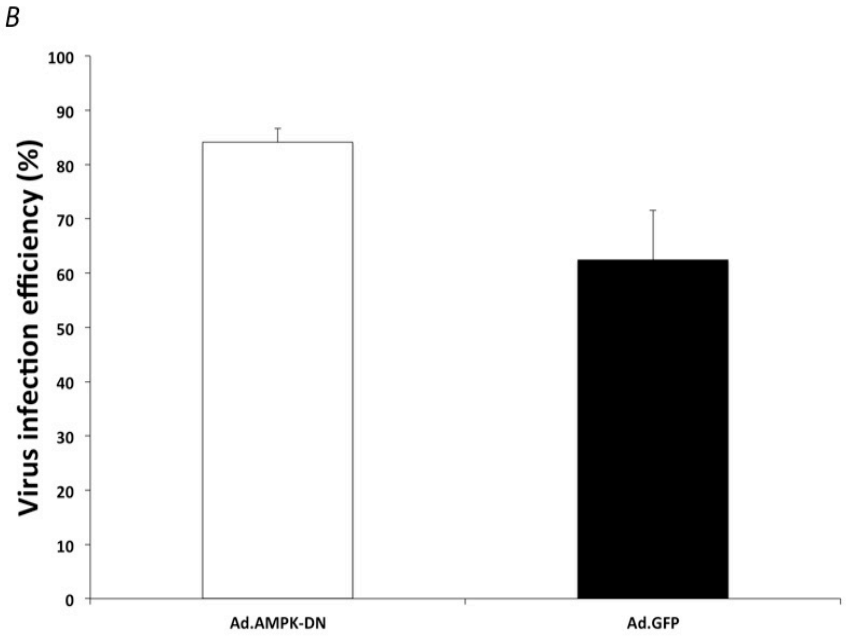
PC3 cells were seeded 24 h before siRNA transfection. *PRKAA1* and non-coding (NT) control siRNA were transfected using Lipofectamine RNAiMAX for 72 h at a concentration of 10 nM. Cells were then incubated for 2 h in serum-free medium before incubation in the presence or absence of (A) AICAR or (B) A769662. EGF (20 ng/mL) was added 15min prior to lysates preparation. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with antibodies indicated. GAPDH was used as loading control. Representative blots are shown (N=3).

3.2.5 Down-regulation of AMPK using adenoviruses expressing a dominant negative AMPK mutant in prostate cancer cell lines

Since siRNA-mediated down-regulation of AMPK was not sufficient to eliminate AMPK activity, infection with adenoviruses expressing a myc-tagged DN AMPK α1 mutant (Ad.AMPK-DN) (Woods *et al.*, 2000) was optimised in PC3 and DU145 cell lines in the view to establish a better system to down-regulate stimulated AMPK activity. Adenoviruses expressing GFP (Ad.GFP) were used as a control. Virus infection was firstly assessed using Confocal microscopy with acceptable infection efficiency in both PC3 (>60%) and DU145 (>80%) cells. However, infection of PC3 cells with 200 IFU/cell Ad.AMPK-DN did not abolish AICAR-stimulated AMPK activation compared to Ad.GFP-infected cells as assessed by immunoblotting, although reduced phospho-ACC Ser79 was noticed in Ad.AMPK-DN infected cells. Similarly, infection of DU145 cells with 100 IFU/cell Ad.AMPK-DN did not abolish AICAR-stimulated AMPK activation compared to Ad.GFP-infected cells as assessed by immunoblotting, although reduced phospho-ACC Ser79 was noticed in Ad.AMPK-DN infected cells (Figures 3.12 and 3.13).

It is apparent that down-regulation of stimulated AMPK activity *in vitro* was very difficult to achieve after a modest effect using both siRNA or adenoviruses. Therefore the AMPK activity in clinical PC samples was assessed to better understand the potential role of AMPK *in vivo*.


A
control
Ad.AMPK-DN
Ad.GFP
GFP
Alpha 1
Phalloidin
RedDot
merge


B
Virus infection efficiency (%)
100
90
80
70
60
50
40
30
20
10
0
Ad.AMPK-DN
Ad.GFP

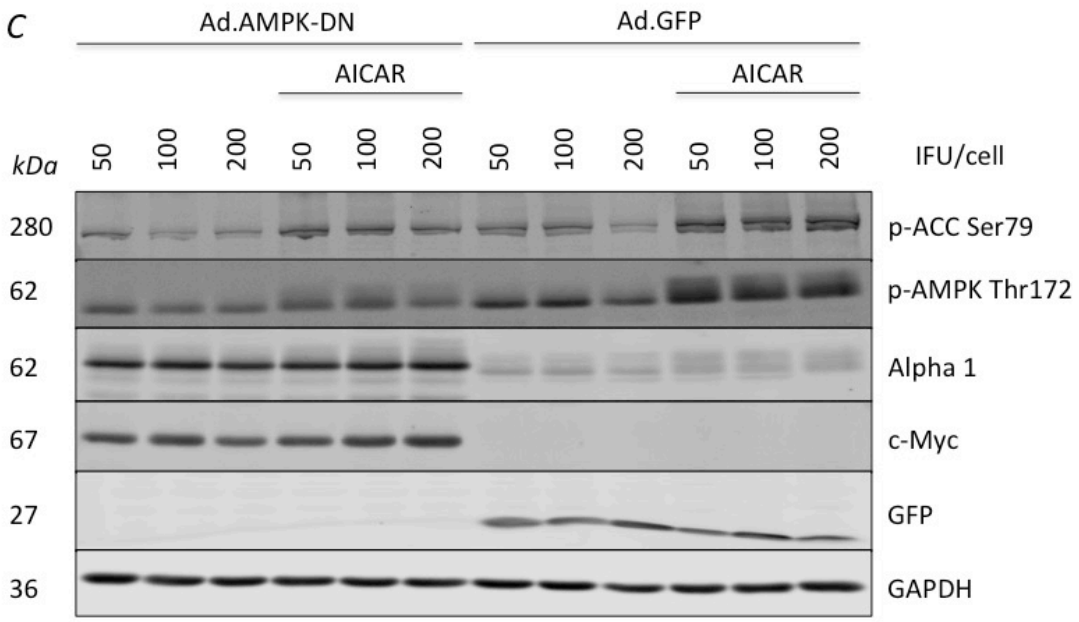
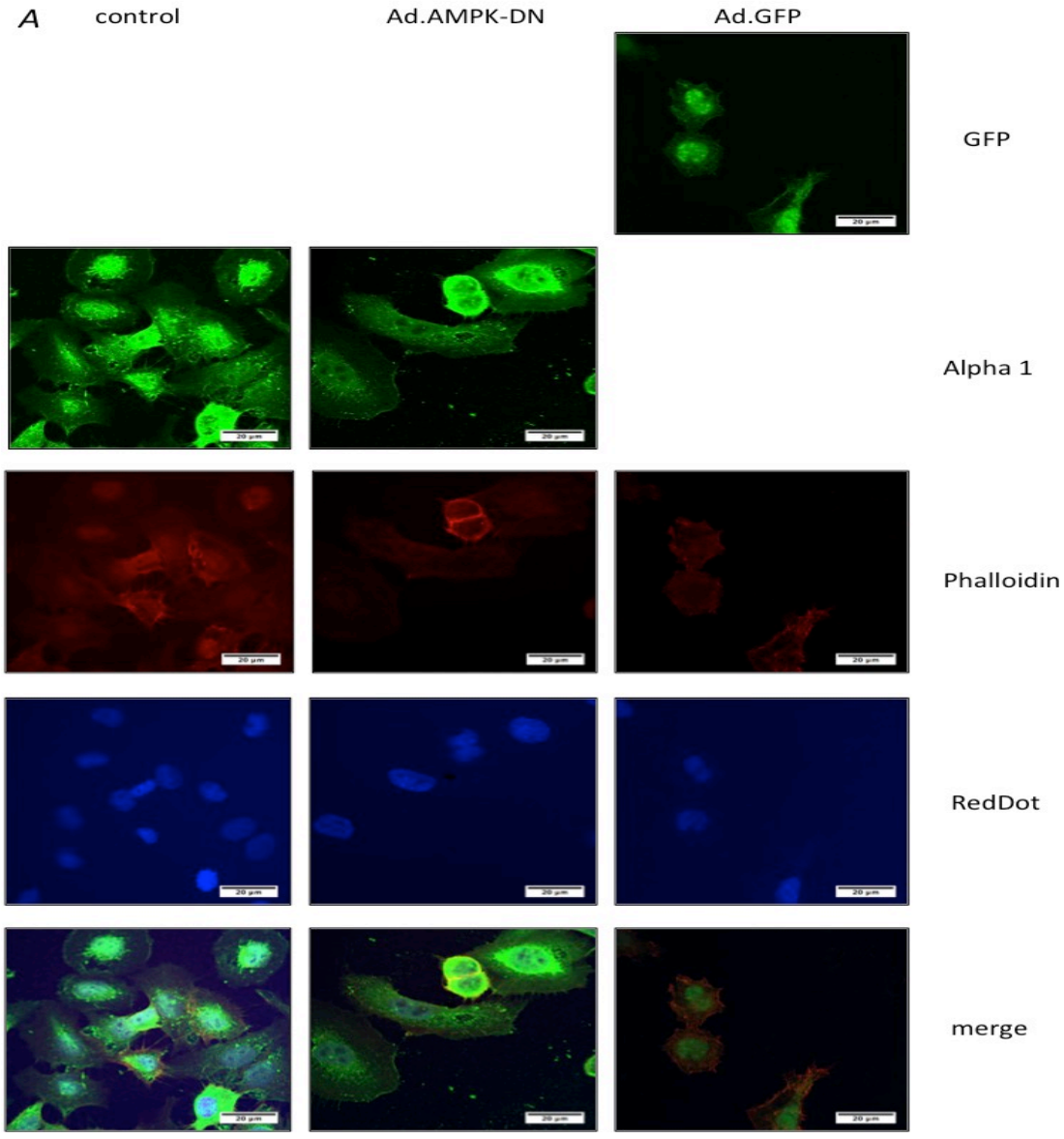
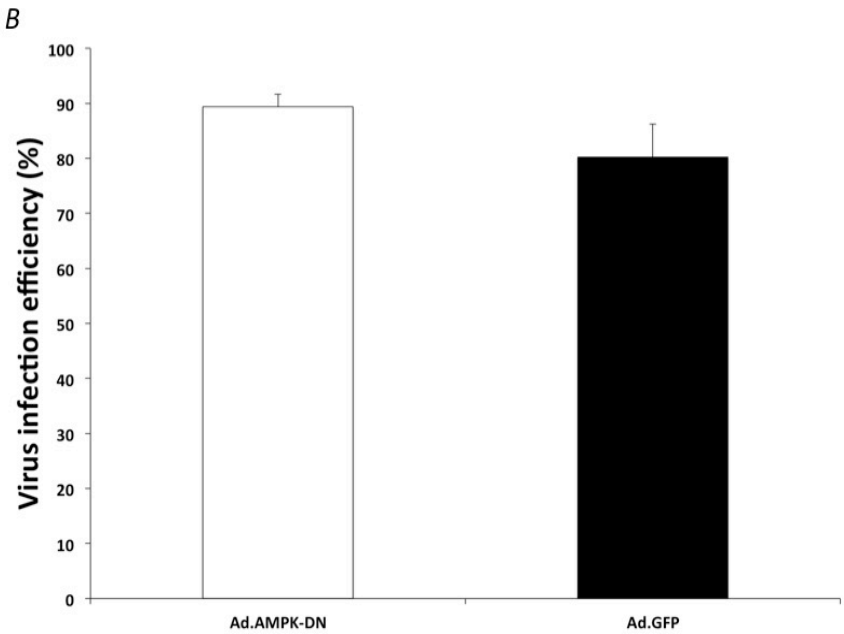


Figure 3.12 Effect of infection with Ad.AMPK-DN on AICAR-stimulated AMPK activation in PC3 cells

(A) Confocal microscopy analyses of virus infection efficiency: PC3 cells were seeded in a 12-well plate with sterile cover slips overnight allowing for attachment. The cells were infected with Ad.AMPK-DN or Ad.GFP adenoviruses (200 IFU/cell) and incubated for 3 h in serum-free medium before incubated with full medium for 48 h. The cover slips were then washed and fixed using 3% paraformaldehyde. The cover slips were immunofluorescence labelled as described in Chapter 2.2.13 and images were taken using Confocal microscopy as described in Chapter 2.2.14, 10 fields/slide were analysed. For Ad.GFP, 4 slides were analysed. For Ad.AMPK-DN, 3 slides were analysed. (B) Virus infection efficiency was assessed by Confocal microscopy (N=1). (C) PC3 cells were infected with Ad.AMPK-DN or Ad.GFP adenoviruses at different IFU as indicated for 48 h before incubation with 1 mM AICAR for 2 h and lysates prepared. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated (N=1). Reduced AICAR-stimulated phospho-ACC Ser79 is observed in Ad.AMPK-DN compared to Ad.GFP at 200 IFU/cell. c-Myc and GFP were used as quality control for Ad.AMPK-DN and Ad.GFP, respectively. GAPDH was used as loading control.


A
control
Ad.AMPK-DN
Ad.GFP
GFP
Alpha 1
Phalloidin
RedDot
merge


B
Virus infection efficiency (%)
100
90
80
70
60
50
40
30
20
10
0
Ad.AMPK-DN
Ad.GFP

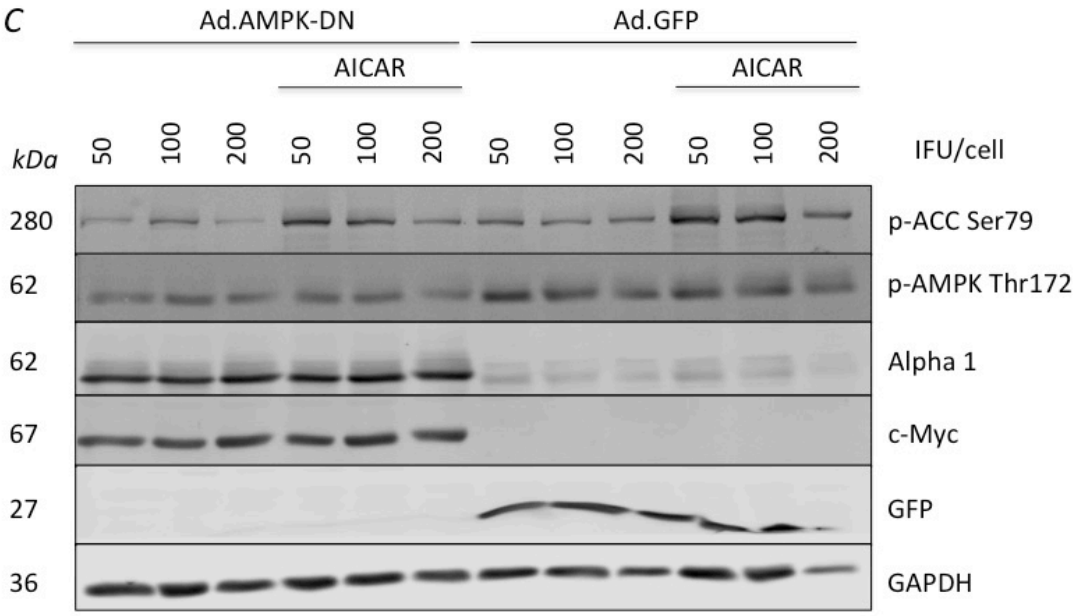


Figure 3.13 Effect of infection with Ad.AMPK-DN on AICAR-stimulated AMPK activation in DU145 cells

(A) Confocal microscopy analyses of virus infection efficiency: DU145 cells were seeded in a 12-well plate with sterile cover slips overnight allowing for attachment. The cells were infected with Ad.AMPK-DN or Ad.GFP adenoviruses (100 IFU/cell) and incubated for 3 h in serum-free medium before incubated with full medium for 48 h. The cover slips were then washed and fixed using 3% paraformaldehyde. The cover slips were immunofluorescence labelled as described in Chapter 2.2.13 and images were taken using Confocal microscopy as described in Chapter 2.2.14, 10 fields/slide were analysed. For Ad.GFP, 4 slides were analysed. For Ad.AMPK-DN, 3 slides were analysed. (B) Virus infection efficiency was assessed by Confocal microscopy (N=1). (C) DU145 cells were infected with Ad.AMPK-DN or Ad.GFP adenoviruses at different IFU as indicated for 48 h before incubation with 1 mM AICAR for 2 h and lysates prepared. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated (N=1). Reduced AICAR-stimulated phospho-ACC Ser79 is observed in Ad.AMPK-DN compared to Ad.GFP at 100 IFU/cell. c-Myc and GFP were used as quality control for Ad.AMPK-DN and Ad.GFP, respectively. GAPDH was used as loading control.

3.2.6 The status of AMPK activity in clinical prostate cancer samples

A tissue micro array (TMA) consisting of both benign prostate hyperplasia (BPH) and PC were analysed by Dr Yashmin Choudhury and Dr Imran Ahmad (University of Glasgow) using immunohistochemistry as described in 2.2.15 (Choudhury *et al.*, 2014). PC samples exhibited increased levels of both phospho-AMPK Thr172 and phospho-ACC Ser79 when compared to BPH samples. Results also suggested a trend of increased phospho-AMPK in tumours with higher Gleason score. In some samples, significant phospho-ACC is observed despite a low phospho-AMPK level (Figure 3.14).

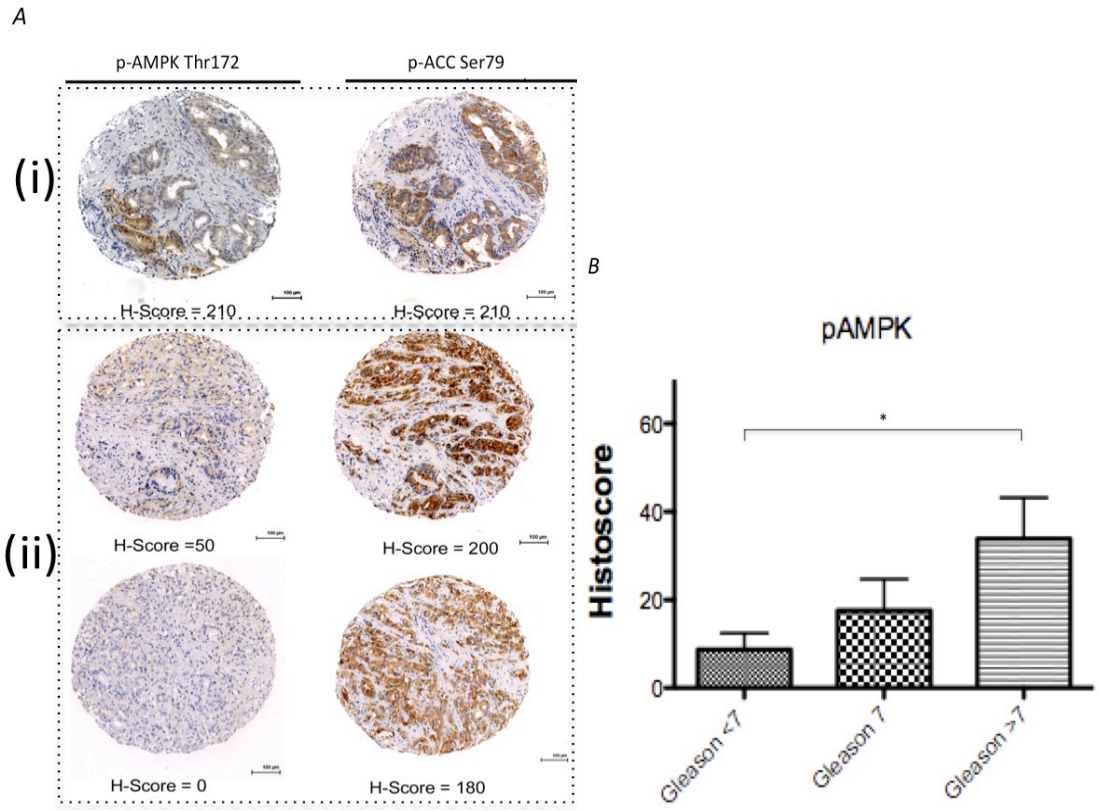


Figure 3.14 Analysis of AMPK phosphorylation in clinical prostate cancer samples

Immunohistochemical analysis using TMA in clinical prostate cancer samples, (A) (i) high H-Score observed for both phospho-AMPK Thr172 and phospho-ACC Ser79 and (ii) low H-score for phospho-AMPK Thr172 but high H-score for phospho-ACC Ser79, scale bar represents 100 μm, (B) The trend of increasing phospho-AMPK Thr172 with higher Gleason score (*: $p<0.05$, Mann-Whitney test). This analysis was performed by Dr Yashmin Choudhury and Dr Imran Ahmad (University of Glasgow).

This figure is reproduced under the Creative Commons Attribution License using Figure 1F and 1G from article "AMP-activated protein kinase (AMPK) as a potential therapeutic target independent of PI3K/Akt signaling in prostate cancer" by Choudhury et al, Oncoscience (2014); 1(6) 446-456.

3.3 Discussion

Initially, six PC cell lines were used in a pilot experiment to assess basal AMPK expression and activity along with the expression of the two AMPK kinases LKB1 and CaMKK2. These cell lines included two AR-dependent cell lines; CWR22 and LNCaP and four AR-independent cell lines; PC3, PC3M, DU145 and LNCaP-AI. These cell lines have different properties in terms of expression of AR (Tilley *et al.*, 1990, Newmark *et al.*, 1992), LKB1 and CaMKK2 (Table 3.1). The data suggest that there are differences between basal AMPK activity across the six PC cell lines used. Although this cannot predict the AMPK activity *in vivo*, it is still interesting that the activity of AMPK is markedly different in PC3 and PC3M cells despite the similar level of AMPK expression with PC3M having higher phospho-AMPK levels compared to PC3. The status of the two upstream kinases might explain this finding given the similar expression of LKB1 in both cell lines, but higher CaMKK2 expression in PC3M cells. It is also possible that AR plays a significant role, given that AR is not only important in PC carcinogenesis, but also has been reported to regulate CaMKK2 signalling (Karacosta *et al.*, 2012, Shima *et al.*, 2012). The analysis of AMPK subunit isoform expression in PC3, DU145 and LNCaP provides evidence that different PC cells may have different AMPK subunit isoforms, with PC3 and LNCaP cells expressing higher levels of AMPK α2 than DU145 cells, and LNCaP cells expressing higher levels of AMPK β2 compared to PC3 and DU145 cells. This is very helpful in understanding PC cell biology as different AMPK complexes containing α subunit isoforms have been reported to have different substrate specificity and subcellular localisation (Woods *et al.*, 1996b, Salt *et al.*, 1998) and some AMPK activators (A769662, salicylate) have been reported to only activate complexes containing β1 isoforms (Scott *et al.*, 2008). However, care should be taken when interpreting these data as the antibodies used in those experiments exhibited substantial non-specific species. In addition, the results shown here also confirmed a lack of LKB1 in DU145 cells as described preciously by others (Yun *et al.*, 2005).

Three PC cell lines; PC3, DU145 and LNCaP were chosen to carry out further experiments based on their different expression profiles as described above.

AMPK could be activated by either AICAR or A769662 in PC3, DU145 and LNCaP cells as assessed by phosphorylation of ACC. Although a slightly different incubation time was found to achieve maximal stimulation for each activator in a given cell line, optimal concentrations for both AMPK activators were the same in all three cells. Concentrations lower than 1 mM AICAR have been used in previous studies in PC cell lines, however, in those studies longer incubation times were used (Xiang *et al.*, 2004, Yun *et al.*, 2005, Isebaert *et al.*, 2011). A lower concentration of A769962 was also used for a longer incubation period in previous study in PC3 cells (Chen *et al.*, 2011). Because the activation of AMPK by AICAR relies on LKB1 (Sakamoto *et al.*, 2005), the significant AICAR-stimulated phosphorylation of ACC in DU145 cells may be due to allosteric activation of AMPK given that the phospho-AMPK Thr172 is not increased in line with phosphorylation of ACC. Moreover, because A769662 has been reported to only activate AMPK complexes containing the β1 isoform (Scott *et al.*, 2008), this also suggests all the three cell lines used in these experiments contain the β1 isoform in agreement with the subunit isoform data presented in Figure 3.2. This is the first characterisation of the time-course and concentration-dependence of AMPK activation by AICAR and A769662 in these PC cell lines.

Optimisation of down-regulation of AMPK activity using AMPK α1 siRNA targeting the *PRKAA1* gene in PC3 and DU145 was performed. Initially, siRNA knockdown was not satisfactory despite using different siRNA from different suppliers and different approaches including HiPerFect (Qiagen) and Nucleofection (Lonza). The Lipofectamine RNAiMAX (Life Technologies) approach with ON-TARGETplus siRNA (Thermo Scientific) finally achieved *PRKAA1* gene knockdown in PC3 cells. However, the activity of AMPK is still sufficient to demonstrate substantial phosphorylation of ACC upon stimulation with AICAR or A769662. These results suggest that knockdown of one catalytic subunit isoform of AMPK is not sufficient to abolish its function in PC cells. An alternative explanation is that even with such a substantial knockdown in terms of AMPK α1 expression (80%), the remaining 20% of the protein is still sufficient to phosphorylate ACC.

Adenoviruses expressing DN AMPK α1 mutant were utilised as an alternative tool to attenuate AMPK activity. Optimisation was performed using Ad.AMPK-DN, which expresses a DN myc-tagged AMPK α1 mutant. Ad.GFP was used as a negative control for cells infected with Ad.AMPK-DN. The optimum infections were achieved in PC3 is with 200 IFU/cell and in DU145 with 100 IFU/cell, which correlates well with a recent study using the same virus loading in both cell lines (Pei *et al.*, 2014). AICAR stimulated ACC phosphorylation level was markedly decreased in Ad.AMPK-DN infected cells compared to Ad.GFP infected cells.

Previously, one study had shown that human PC tissue exhibits higher AMPK activity (as measured by phospho-ACC Ser79) compared to normal prostate tissue (Park *et al.*, 2009). However, no correlation between AMPK activity and Gleason score was established in that study (Park *et al.*, 2009). The current study provided first such evidence that AMPK activity is linked to Gleason score, although the underlying mechanism remains unclear. It is possible that when PC progresses, more energy is utilised, leading to increased AMPK activity. However, increased AMPK activity could also be explained as a protective measure against tumourigenesis. Therefore, extra care should be taken when interpreting any such data in this context. In addition, the disassociation between phospho-ACC and phospho-AMPK is unexplained in some of the clinical samples examined.

Overall, this chapter provided important basic characteristics of AMPK-associated molecular biology in PC. Using two structurally-unrelated AMPK activators, AICAR and A769662, an *in vitro* system was established in three PC cell lines (PC3, DU145 and LNCaP) as tools to explore AMPK-related functions in PC. Optimisation of down-regulation of AMPK was also attempted using different experimental approaches, which could be further refined for use in studies of AMPK function in PC. Preliminary data using clinical PC samples demonstrated a positive association of AMPK activity with PC severity. It would be both interesting and important to know what effect AMPK has in PC cellular function such as proliferation and migration.

Chapter 4. The role of AMPK activation in human prostate cancer cell proliferation and migration

4.1 Introduction

4.1.1 The role of AMPK activation in prostate cancer cell proliferation

Proliferation has long been recognised as an important process in carcinogenesis (Bresciani, 1968). Previously, several studies have examined the effect of AMPK activators on prostate cancer (PC) cell proliferation. At the start of this study, there were contrasting data regarding the role of AMPK in PC proliferation. Cell growth was reported to be inhibited after treatment with AICAR as assessed by cell counting, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and BrdU uptake assays in PC3 cells (Xiang *et al.*, 2004). In DU145 cells, AICAR incubation reduced cell number (Xiang et al., 2004), an effect later reported to be concentration-dependent (Sauer *et al.*, 2012). Metformin was similarly reported to reduce cell proliferation in PC3, DU145 and LNCaP cells measured by both cell counting and MTT assay (Ben Sahra *et al.*, 2008, Ben Sahra *et al.*, 2010a). The effect of metformin on PC3 cell proliferation, assessed using a colourimetric assay, has also been reported to be concentration-dependent manner (Zakikhani *et al.*, 2008). In contrast, down-regulation of AMPK catalytic subunits by small interfering RNA or incubation with compound C (dorsomorphin), a potent AMPK inhibitor, decreased cell proliferation, as measured by cell counting, in LNCaP and 22Rv1 cells (Park *et al.*, 2009). In all of these studies, the AMPK activators utilised (mostly AICAR or metformin) were not highly specific. It has been reported that the more specific AMPK activator, A769662, could inhibit cancer growth *in vitro* and *in vivo* (Huang *et al.*, 2008), yet whether A769662 has anti-proliferative actions in PC cell lines had not been explored.

4.1.2 The role of AMPK activation in prostate cancer cell migration

Migration is a key factor contributing to metastasis in malignancy including PC (Jacob *et al.*, 1999, Jones *et al.*, 2006). Prior to the start of these studies, there were far fewer studies that had investigated the actions of AMPK activators on PC

cell migration, although it had been reported that overexpression of CaMKK2, one upstream kinase of AMPK, led to increased AMPK phosphorylation and cell migration in LNCaP cells (Frigo *et al.*, 2011). In addition, adiponectin was demonstrated to stimulate AMPK phosphorylation and increase migration of PC3, DU145 and LNCaP cells in a manner sensitive to compound C or siRNA targeted to AMPK (Tang and Lu, 2009). However, in contrast, inactivation of AMPK using a DN mutant in C4-1 cells was also reported to increase cell migration (Zhou *et al.*, 2009). As mentioned above, A769662 has been reported to inhibit cancer growth *in vitro* and *in vivo* (Huang *et al.*, 2008), yet whether A769662 has anti-migratory actions in PC cell lines had not been explored.

This study therefore examined the effects of A769662 on PC cell proliferation and migration, comparing those effects with AICAR, which activates AMPK by an indirect mechanism (Chapter 1.2.3).

4.2 Results

4.2.1 The effect of AMPK activators on prostate cancer cell viability

Initially, the effect of AMPK activators on viability was assessed in both PC3 and DU145 cells using the WST-1 viability assay, which measures cell metabolism. Both AICAR and A769662 significantly decreased cell viability in both PC3 and DU145 cells. AICAR (1 mM) inhibited PC3 and DU145 cell viability by 80% and 70% respectively, whereas A769662 (100μM) inhibited by 80% and 40% respectively (Figure 4.1). To examine whether these effects of AICAR and A769662 were AMPK dependent, the viability assay was repeated in PC cell lines infected with Ad.AMPK-DN adenoviruses. Infection with Ad.AMPK-DN has no effect on the inhibition of PC3 and DU145 cell viability produced by AICAR or A769662 compared to cells infected with Ad.GFP or uninfected cells, although infection with adenoviruses reduced DU145 cell viability. (Figure 4.2) To further examine the effect of AMPK activators on cell viability, isogenic cell lines PC3 and PC3M were used. The inhibitory effects of both AICAR and A769662 on viability are concentration-dependent in PC3 and PC3M cells. Under same concentration of AICAR, viability was decreased more in PC3 cells than PC3M cells. The inhibitory effect produced by A769662, in the other hand, was similar in both PC3 and PC3M cells (Figure 4.3). Moreover, the inhibitory effect of 500μM AICAR was slightly reversed by compound C, a chemical inhibitor of AMPK. Compound C, however, had no effect on the inhibitory effect produced by 2 mM AICAR in both PC3 and PC3M cells. Down-regulation of AMPK α1 using siRNAs targeting *PRKAA1* also produced a reversal effect on the inhibition of viability induced by 2 mM AICAR in both PC3 and PC3M cells, although these reversal effects are not statistical significant. (Figure 4.3) The observation in these viability experiments raised a question as whether the inhibitory effects of AMPK activators truly reflect changes in cell proliferation or rather are a result of metabolic changes. Another approach to accurately measure cell proliferation was therefore used subsequently.

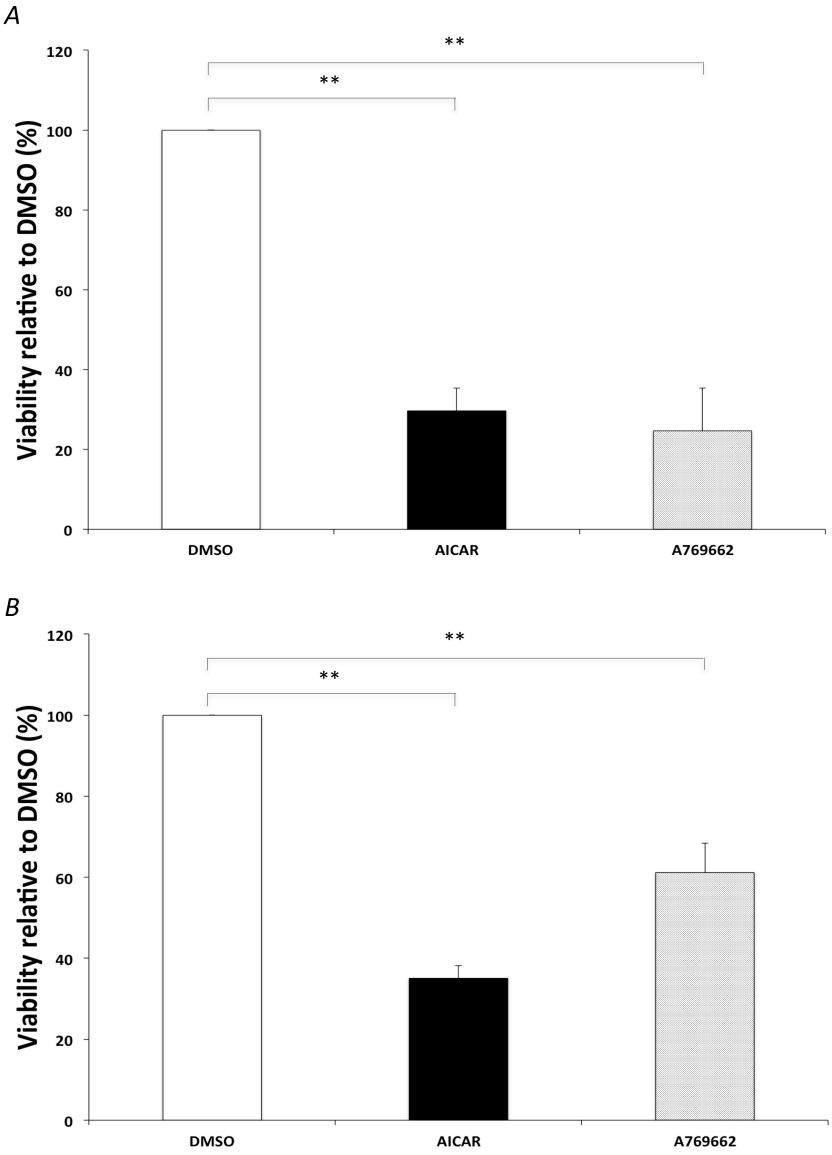


Figure 4.1 Viability assay of PC3 and DU145 cells 72 h after incubation with AMPK activators

(A) PC3 or (B) DU145 cells were seeded in 96-well plates and incubated for 24 h to allow attachment. The cells were incubated for 2 h in serum-free medium before incubation in the presence or absence of 1 mM AICAR or 100 μM A769662 for 72 h. WST-1 (10% v/v) was added to each well and normalised absorbance at 595 nm was assessed after 120 min. Cell viability was normalised to DMSO control. **: $p<0.01$ compared to DMSO control, experiments were repeated independently for at least three times.

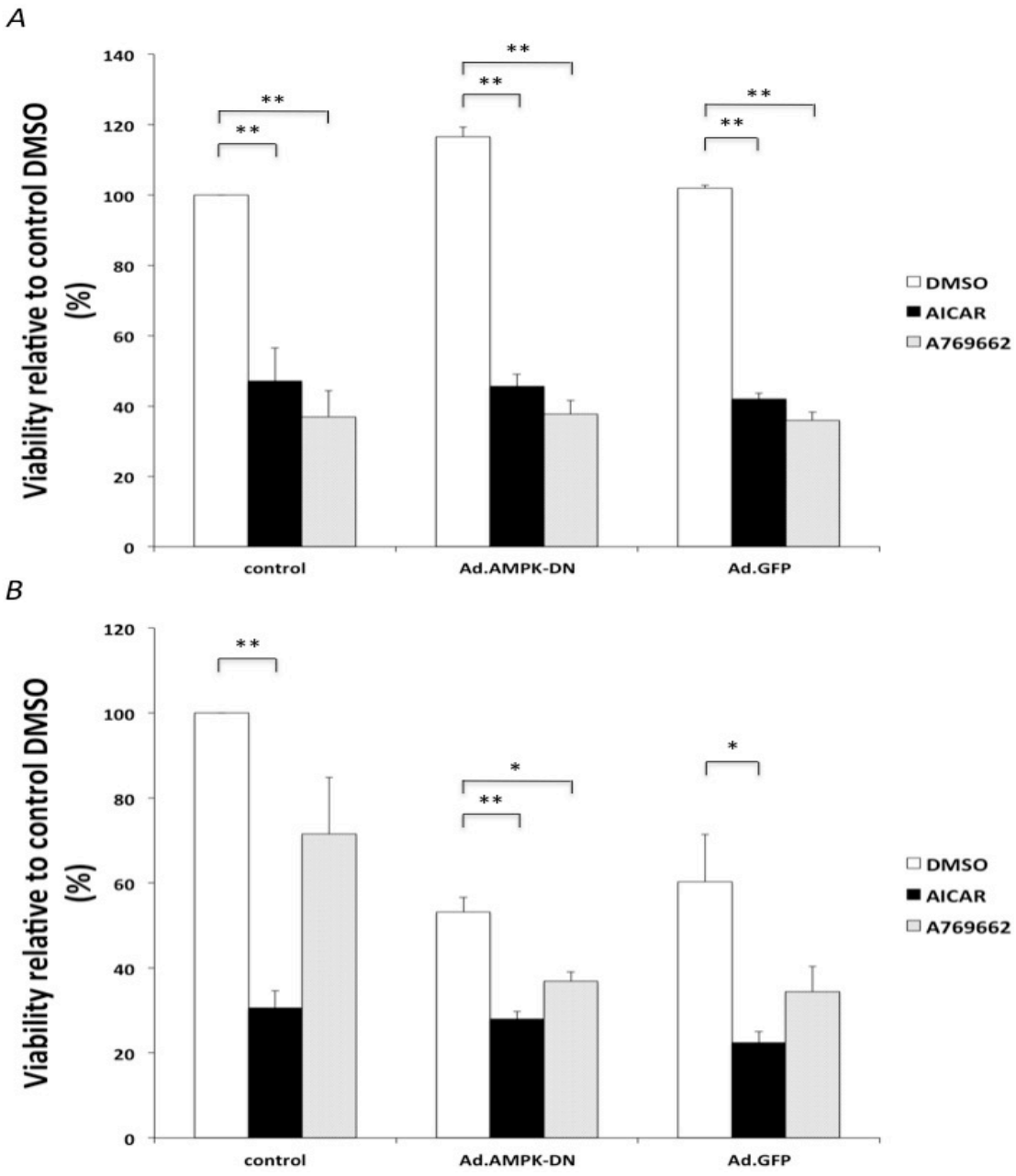


Figure 4.2 Effect of Ad.AMPK-DN adenovirus infection on AICAR and A769662-mediated inhibition of prostate cancer cell viability

Cells were seeded in 96-well plates and incubated for 24 h to allow attachment. The cells were infected with Ad.AMPK-DN or Ad.GFP adenoviruses (200 IFU/cell in PC3, 100 IFU/cell in DU145) and were incubated for 3 h in serum-free medium before incubation in the presence or absence of 1 mM AICAR or 100 μM A769662 for 72 h. WST-1 (10% v/v) was added to each well and normalised absorbance at 595 nm was assessed after 120 min. (A) PC3 cell viability normalised to DMSO control (**: $p<0.01$, N=3). (B) DU145 cell viability normalised to DMSO control (*: $p<0.05$, **: $p<0.01$, N=3).

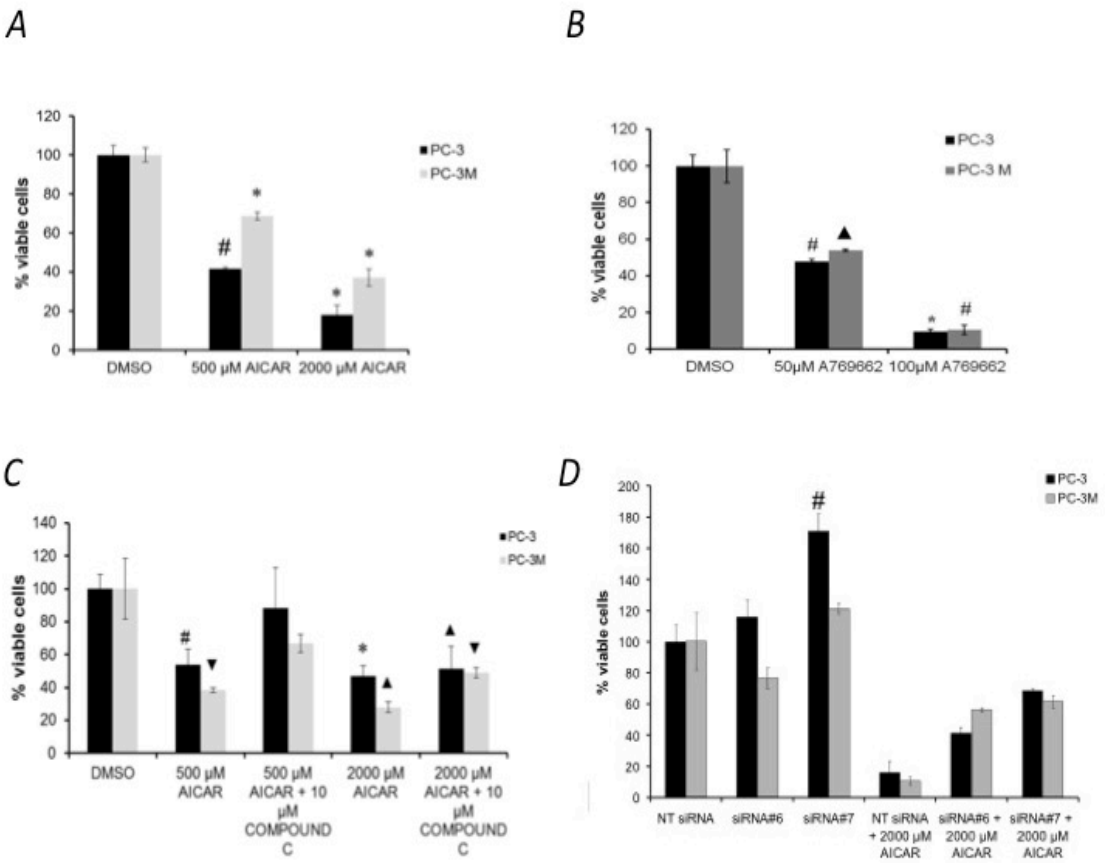


Figure 4.3 Viability assay of PC3 and PC3M cells 72 h after incubation with AMPK activators

PC3 and PC3M cells were seeded in 96-well plates and incubated for 24 h to allow attachment. The cells were incubated for 2 h in serum-free medium before incubation in the presence or absence of (A) 500 μM and 2000 μM AICAR or (B) 50 μM and 100 μM A769662 for 72 h. (C) Viability assay with 2000 μM AICAR and 10 μM compound C alone or in combination for 72 h. (D) Cells were incubated with *PRKAA1* siRNA alone or with 2000 μM AICAR prior to the viability assays. WST-1 (10% v/v) was added to each well and normalised absorbance at 595 nm was assessed after 120 min. Data presented as mean ± SD, *: p≤0.001, #: p≤0.005, ▲: p≤0.01, ▼: p≤0.05 from (A to C: DMSO control, D: NT siRNA control). These experiments were performed by Dr Yashmin Choudhury (University of Glasgow).

4.2.2 The effect of AMPK activators on prostate cancer cell proliferation

The effect of AICAR and A769662 on proliferation was assessed using the BrdU proliferation assay in both PC3 and DU145 cells. In PC3 cells, both AICAR and A7699662 significantly decrease cell proliferation by 40% after 72 h incubation. In DU145 cells however, there is no significant effect observed on BrdU incorporation after 72 h incubation with either AICAR or A769662, although AICAR tended to reduce proliferation (p=0.09) (Figure 4.4). To further assess the AMPK-dependence of the effect of AICAR and A769662 observed in Chapter 4.2.1, mouse embryonic fibroblasts (MEFs) that were wild type (WT) or deficient in *AMPK* $\alpha 1^{-/-}$ and *AMPK* $\alpha 2^{-/-}$ (knock out, KO) were used. These cells were verified to confirm that their genotypes (WT and KO) by immunoblotting prior to the start of the experiments (data not shown) and again in Chapter 6.2.2 (Figure 6.4). AICAR caused a concentration-dependent decrease in proliferation in both WT and *AMPK* $\alpha 1^{-/-}$ *AMPK* $\alpha 2^{-/-}$ KO cells, yet *AMPK* $\alpha 1^{-/-}$ *AMPK* $\alpha 2^{-/-}$ KO cells were more sensitive to AICAR, demonstrating marked inhibition of proliferation at a concentration of 100 μM, whereas 1 mM was required for a similar effect in WT cells (Figure 4.5). A769662 caused a modest inhibition of proliferation in WT cells at a concentration of 30 μM, an effect that was not observed in *AMPK* $\alpha 1^{-/-}$ *AMPK* $\alpha 2^{-/-}$ KO cells. In contrast, 100 μM A769662 markedly inhibited proliferation in both genotypes (Figure 4.6).

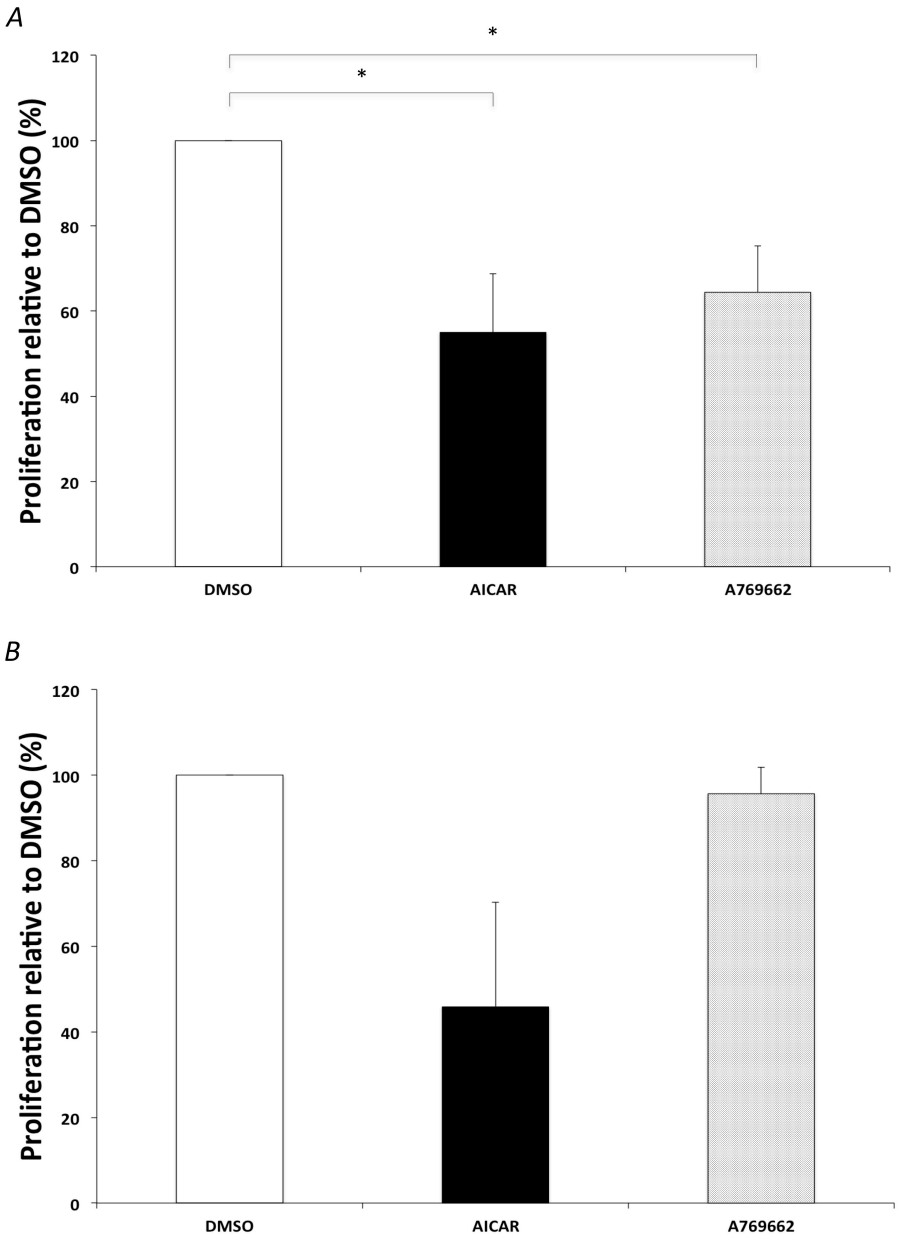


Figure 4.4 Effect of AMPK activators on the proliferation of prostate cancer cell lines

Cells were seeded in 96-well plates and incubated for 24 h to allow attachment. The cells were incubated for 2 h in serum-free medium before incubation in the presence or absence of 1 mM AICAR or 100 μM A769662 for 72 h. BrdU (10% v/v) was added to each well and incubated for a further 2 h. The plate was then fixed and developed according to the protocol and absorbance at 595 nm was assessed. Cell proliferation was normalised to DMSO control. (A) PC3 cells (*: $p<0.05$ compared to DMSO control, N=3). (B) DU145 cells (N=3).

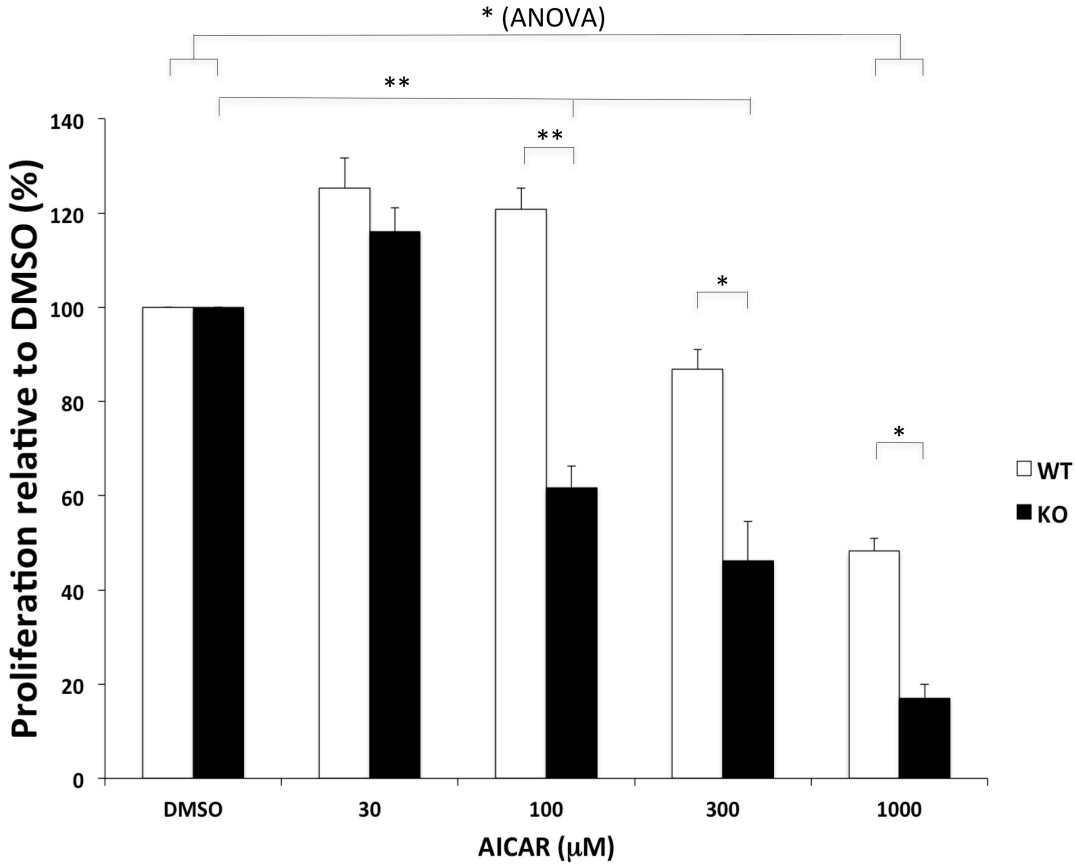


Figure 4.5 Effect of AICAR on cell proliferation of wild type (WT) and *AMPK α1*$^{-/-}$ *AMPK α2*$^{-/-}$ knock out (KO) mouse embryonic fibroblasts

Cells were seeded in 96-well plates and incubated for 2 h to allow attachment. The cells were incubated with AICAR at various concentrations overnight. BrdU (10% v/v) was added to each well and incubated for a further 8 h. The plate was then fixed and developed according to the protocol and BrdU incorporation assessed by absorbance at 595 nm. Cell proliferation was normalised to DMSO control (*: $p<0.05$, **: $p<0.01$, experiments were repeated independently for at least three times).

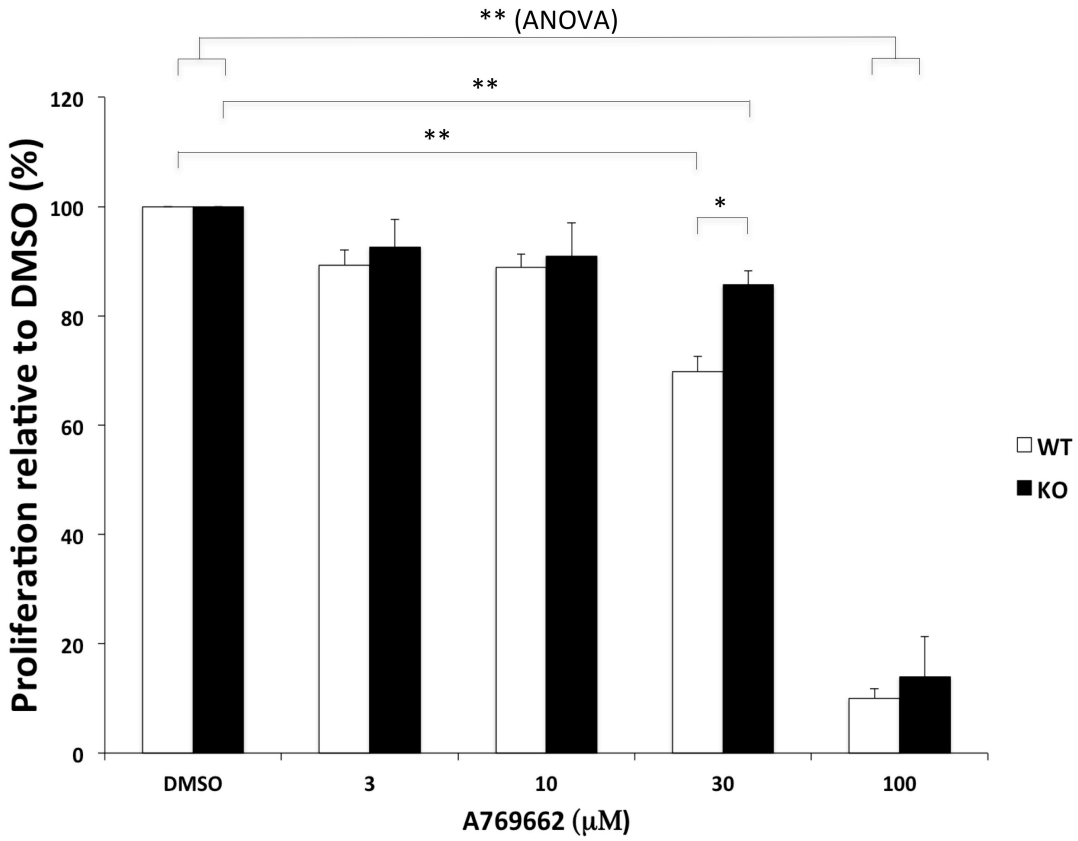


Figure 4.6 Effect of A769662 on cell proliferation of wild type (WT) and *AMPK α1*$^{-/-}$ *AMPK α2*$^{-/-}$ knock out (KO) mouse embryonic fibroblasts

Cells were seeded in 96-well plates and incubated for 2 h to allow attachment. The cells were incubated with A769662 at various concentrations overnight. BrdU (10% v/v) was added to each well and incubated for a further 8 h. The plate was then fixed and developed according to the protocol and BrdU incorporation assessed by absorbance at 595 nm. Cell proliferation was normalised to DMSO control (*: $p<0.05$, **: $p<0.01$, experiments were repeated independently for at least three times).

4.2.3 Effect of AMPK activators on prostate cancer cell migration

4.2.3.1 Effect of AMPK activators on scratch wound healing

PC cell migration was first assessed by an *in vitro* wound healing assay. Incubation with AICAR and A769662 for 22 h inhibited healing of the scratch wound in PC3 cells (Figure 4.7 and Movie 4.1 to 4.4). Yet this effect by AICAR was less marked in DU145 cells, and there is no obvious inhibition by A769662 (Figure 4.8 and Movie 4.5 to 4.8).

4.2.3.2 Cell tracking

More information in cell motility was obtained by tracking cells on the edge of the scratch wound and analysing them for accumulative distance travelled (μm), Euclidean distance travelled (μm) and mean velocity (μm/min). The results showed a trend of decreased motility after incubation with either AICAR or A769662 in PC3 (Figure 4.9 and 4.10) and DU145 cells (Figure 4.11 and 4.12).

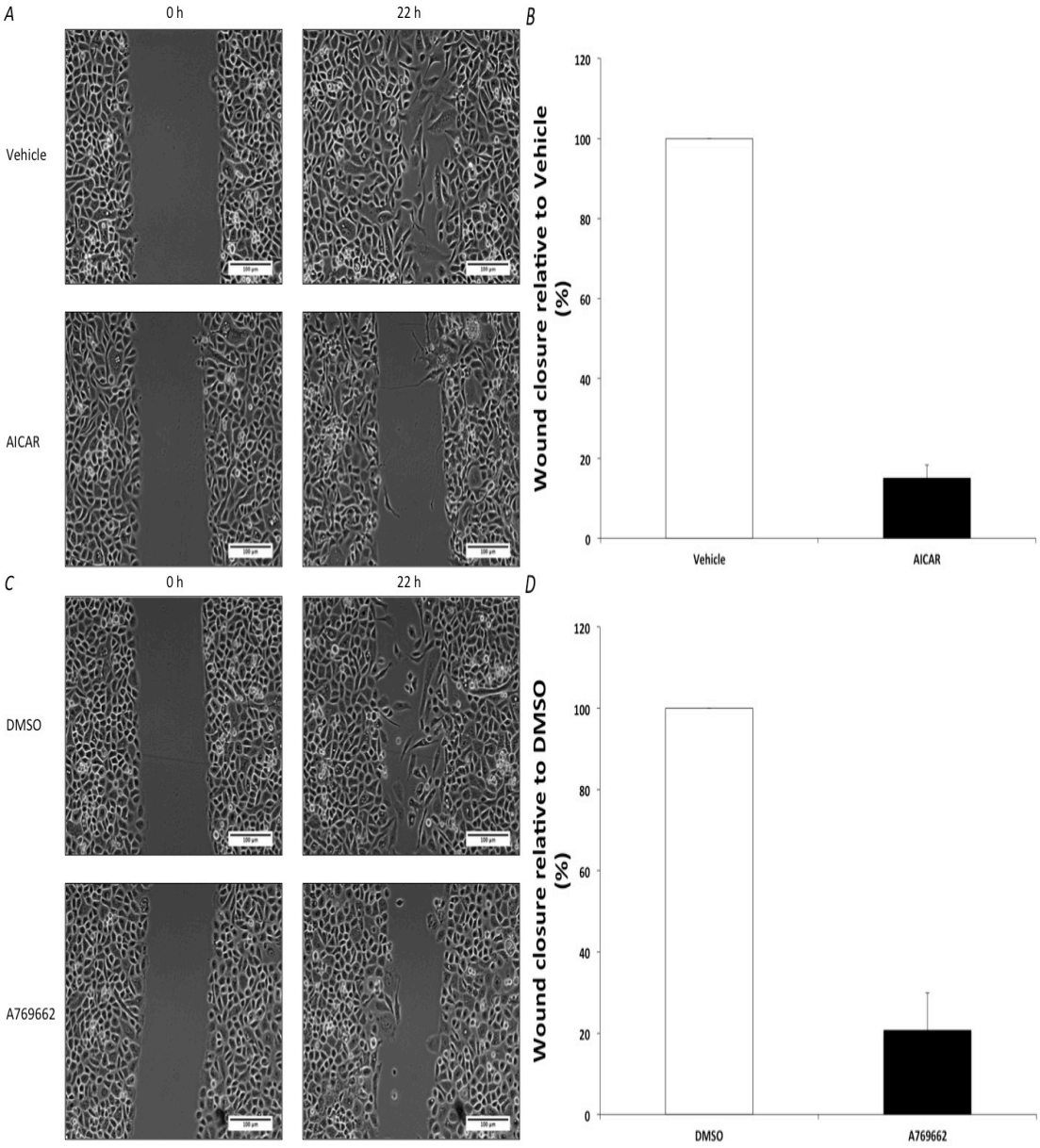


Figure 4.7 Scratch wound healing assay of PC3 cells 22 h after incubation with AMPK activators

Cells were seeded in a 6-well plate with 1% (v/v) FBS for 72 h until fully confluent. The cells were then incubated with serum-free medium for 2 h before being scratched and incubated with 1 mM AICAR or 100 μM A769662 for a further 22 h. Three fields were analysed for each experimental condition. Scale bar represents 100 μm. (A) Representative images for experiment in presence or absence of AICAR with vehicle control. (B) Numerical analysis for AICAR. (C) Representative images for experiment in presence of absence of A769662 with DMSO control. (D) Numerical analysis for experiment in presence or absence of A769662 (N=2).

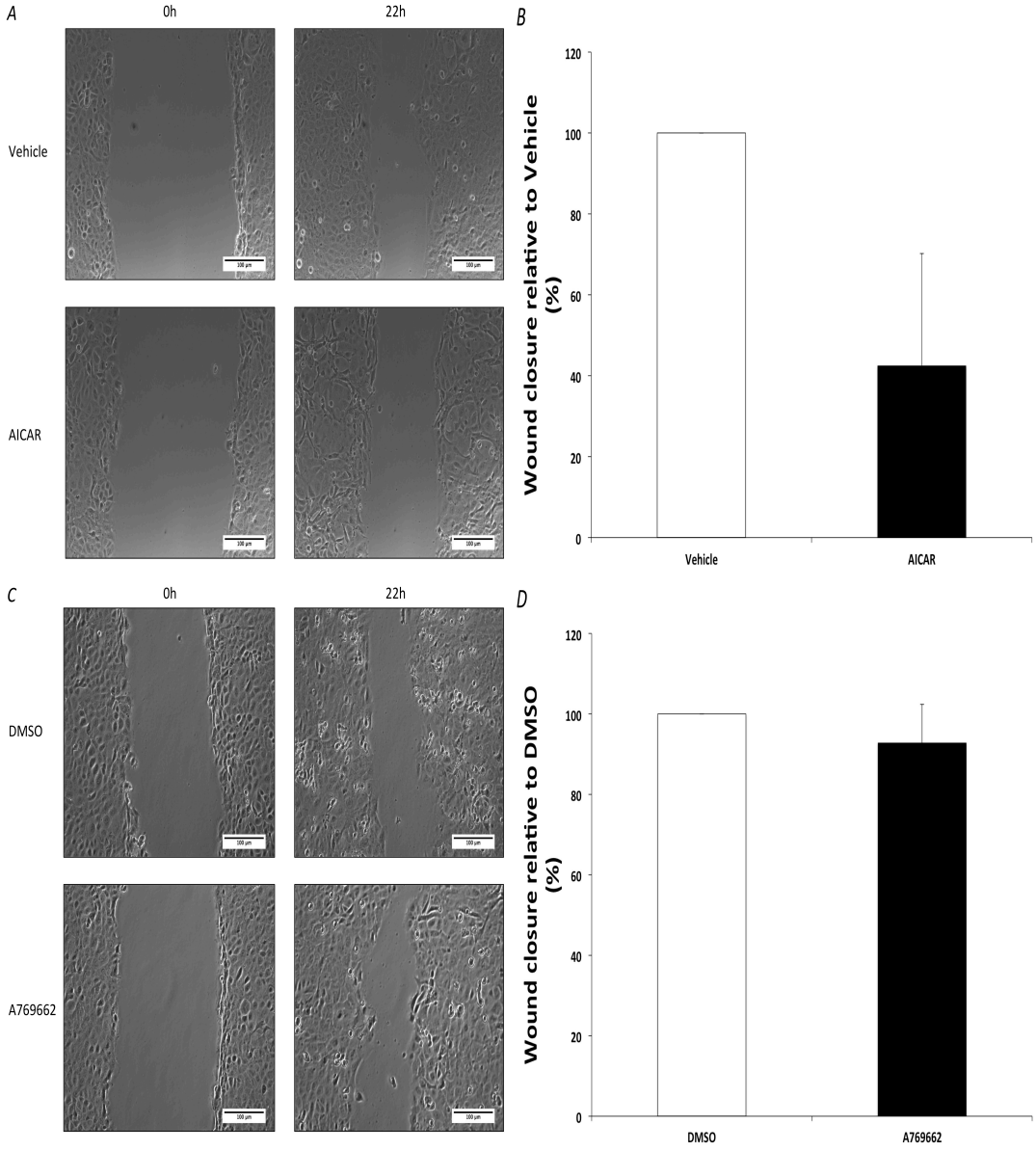


Figure 4.8 Scratch wound healing assay of DU145 cells 22 h after incubation with AMPK activators

Cells were seeded in a 6-well plate with 1% (v/v) FBS for 72 h until fully confluent. The cells were then incubated with serum-free medium for 2 h before being scratched and incubated with 1 mM AICAR or 100 μM A769662 for a further 22 h. Three fields were analysed for each experimental condition. Scale bar represents 100 μm. (A) Representative images for experiment in presence or absence of AICAR with vehicle control. (B) Numerical analysis for AICAR (N=2). (C) Representative images for experiment in presence of absence of A769662 with DMSO control. (D) Numerical analysis for experiment in presence or absence of A769662 (N=2).

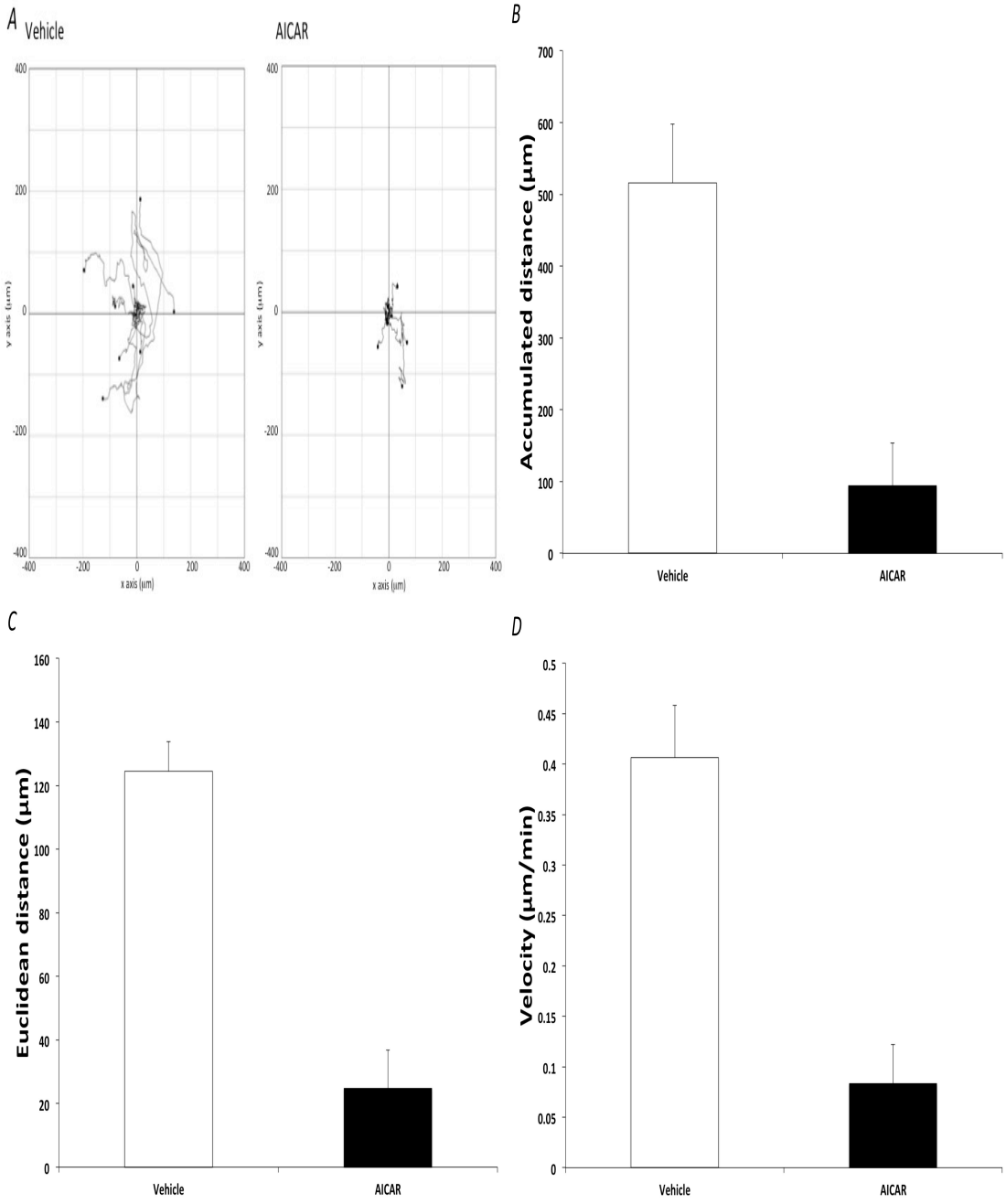


Figure 4.9 Effect of AICAR on PC3 cell motility

Cells were seeded in a 6-well plate with 1% (v/v) FBS for 72 h until fully confluent. The cells were then incubated for 2 h in serum-free medium before incubation with 1 mM AICAR for a further 22 h. Eight cells were tracked for each experimental condition. (A) Spider plot. (B) Accumulated distance analysis (N=1). (C) Euclidean distance analysis (N=1). (D) Velocity analysis (N=1).

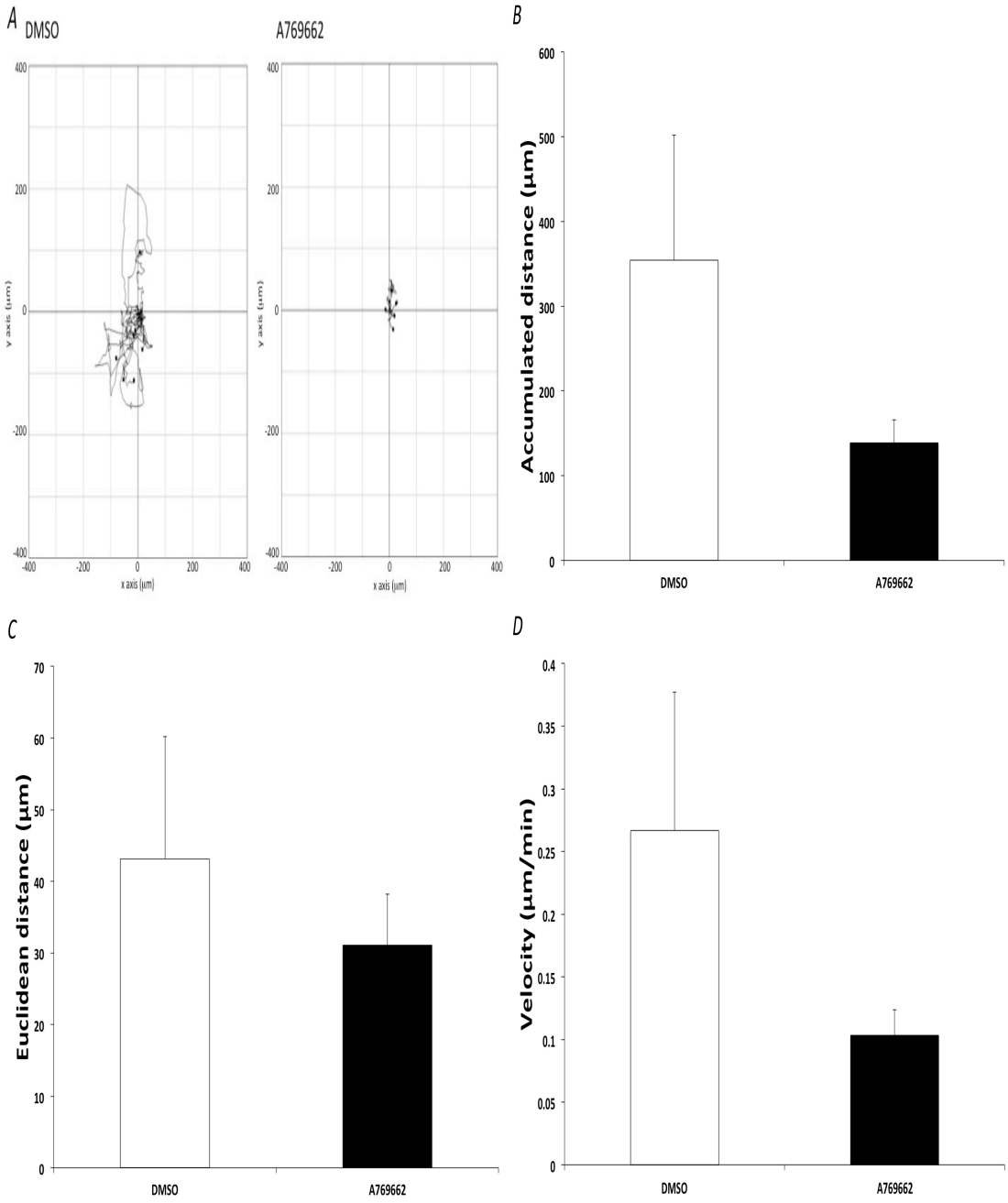


Figure 4.10 Effect of A769662 on PC3 cell motility

Cells were seeded in a 6-well plate with 1% (v/v) FBS for 72 h until fully confluent. The cells were then incubated for 2 h in serum-free medium before incubation with 100 µM A769662 for a further 22 h. Eight cells were tracked for each experimental condition. (A) Spider plot. (B) Accumulated distance analysis (N=1). (C) Euclidean distance analysis (N=1). (D) Velocity analysis (N=1).

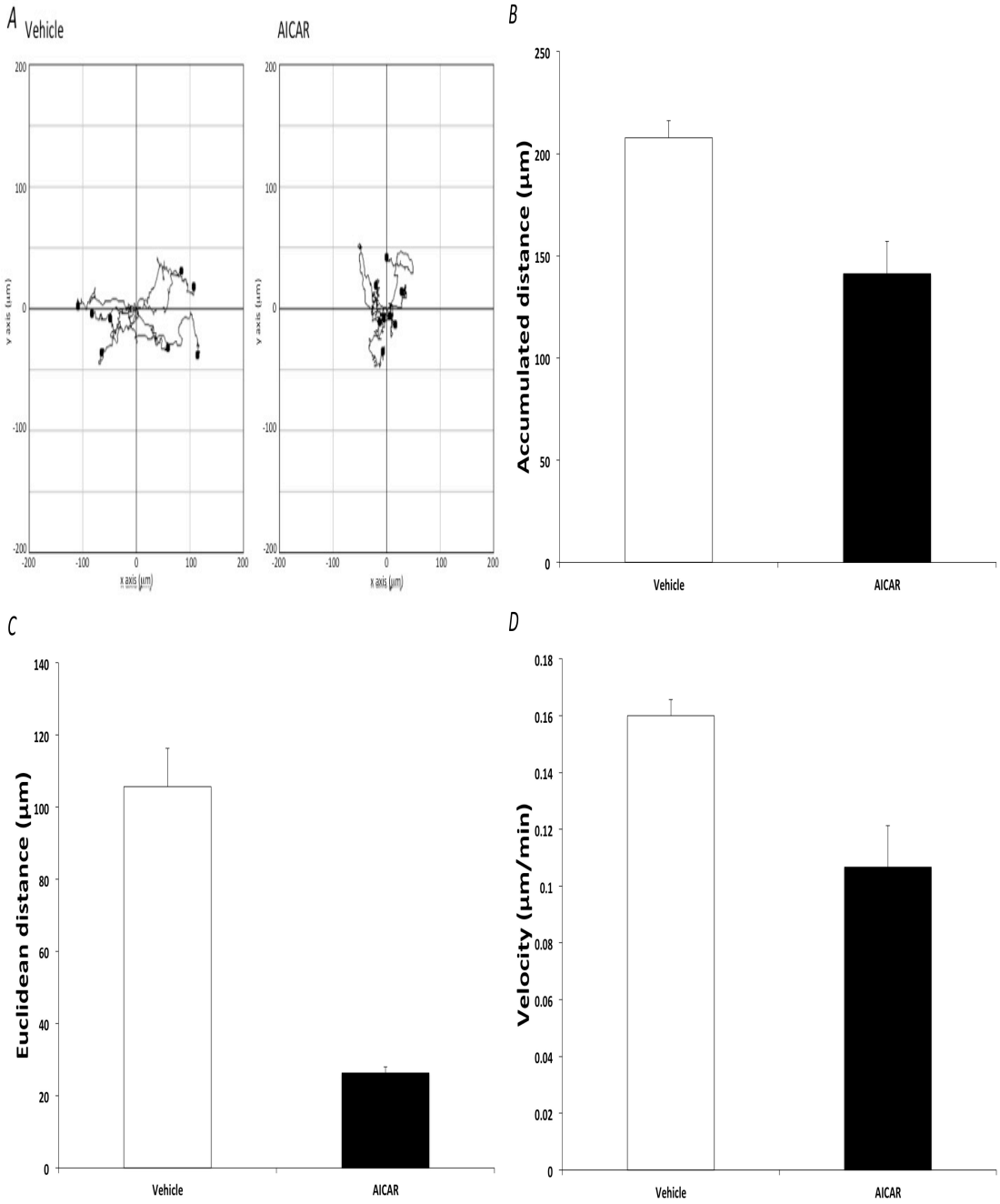


Figure 4.11 Effect of AICAR on DU145 cell motility

Cells were seeded in a 6-well plate with 1% (v/v) FBS for 72 h until fully confluent. The cells were then incubated for 2 h in serum-free medium before incubation with 1 mM AICAR for a further 22 h. Eight cells were tracked for each experimental condition. (A) Spider plot. (B) Accumulated distance analysis (N=1). (C) Euclidean distance analysis (N=1). (D) Velocity analysis (N=1).

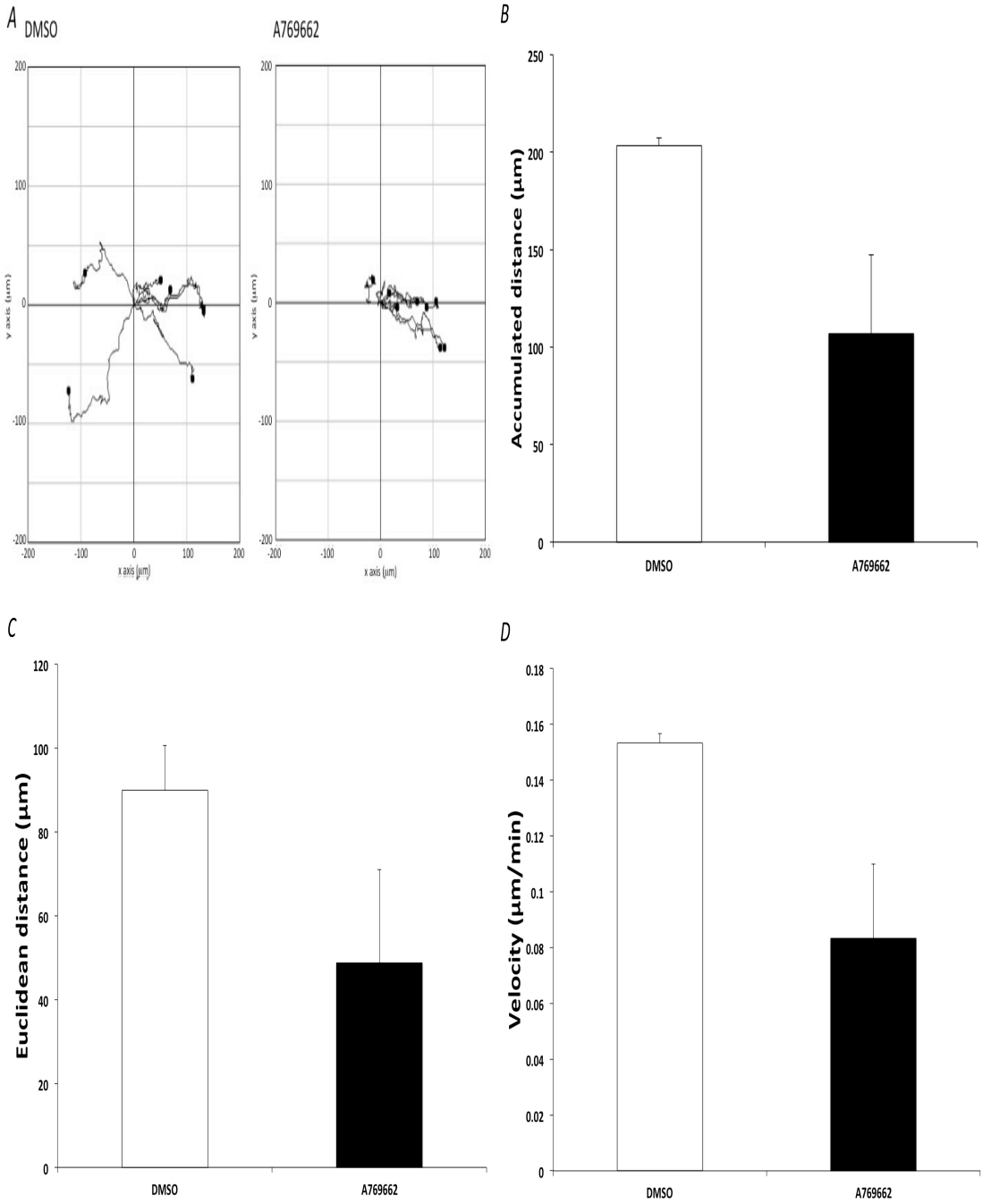


Figure 4.12 Effect of A769662 on DU145 cell motility

Cells were seeded in a 6-well plate with 1% (v/v) FBS for 72 h until fully confluent. The cells were then incubated for 2 h in serum-free medium before incubation with 100 μM A769662 for a further 22 h. Eight cells were tracked for each experimental condition. (A) Spider plot. (B) Accumulated distance analysis (N=1). (C) Euclidean distance analysis (N=1). (D) Velocity analysis (N=1).

4.2.3.3 Effect of AMPK activators on prostate cancer cell line transwell migration

Transwell migration assays were performed to further examine the effect of AICAR or A769662 on chemotaxis in PC3 cells. Interestingly, PC3 cell migration was reduced modestly by AICAR whereas A769662 significantly reduced cell migration (Figure 4.13). In DU145 cells, however, AICAR significantly reduced cell migration whereas A769662 had no effect (Figure 4.14). To examine the AMPK-dependence of this effect, PC3 cells were infected with Ad.AMPK-DN or Ad.GFP. Infection with adenoviruses attenuated migration, yet A769662 still markedly reduced migration in PC3 cells. Interestingly, the anti-migration effect induced by AICAR was no longer statistically significant in Ad.AMPK-DN infected PC3 cells (Figure 4.15). Further experiments using isogenic PC3 and PC3M cells demonstrated that AICAR decreases cell migration in a concentration-dependent manner. Unlike the viability experiments shown in Chapter 4.2.1 (Figure 4.3), AICAR had less effect on PC3 cell migration compared to the more aggressive PC3M cells. Interestingly, *PRKAA1* siRNA could increase cell migration in PC3 cells (Figure 4.16).

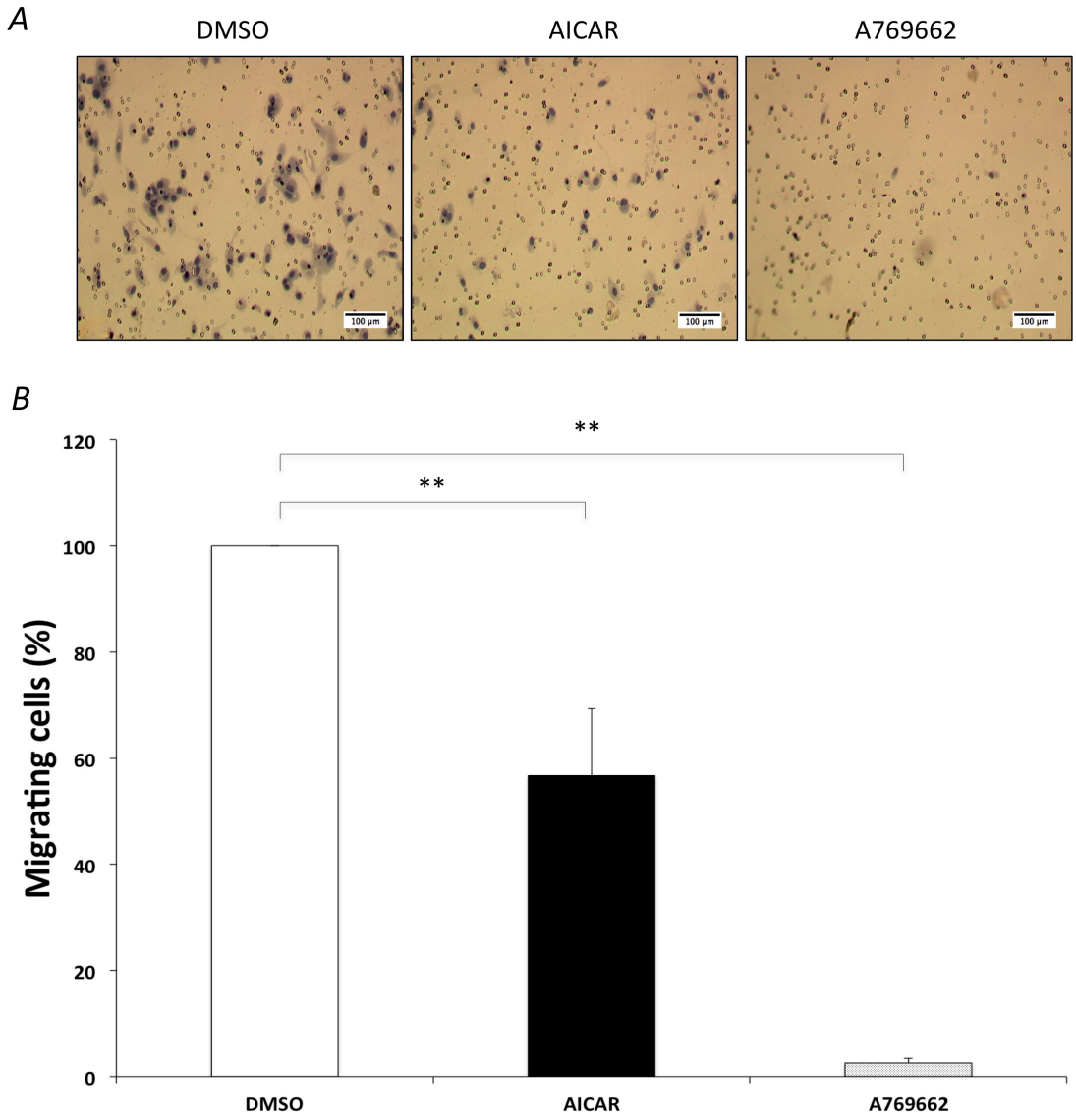


Figure 4.13 Effect of AMPK activators on PC3 cell chemotaxis

PC3 cells were incubated for 24 h in serum-free medium prior to being seeded in 8 μm pore size transwell inserts in serum-free medium. AICAR (1 mM) and A769662 (100 μM) were added in both the transwell inserts and 24-well chambers and incubated for 21 h. The inserts were then fixed with methanol and stained with Haemotaxylin. Four fields were analysed for each replicate. Scale bar represents 100 μm. (A) Representative images. (B) Quantification of migrated cells (**: $p<0.01$, N=6).

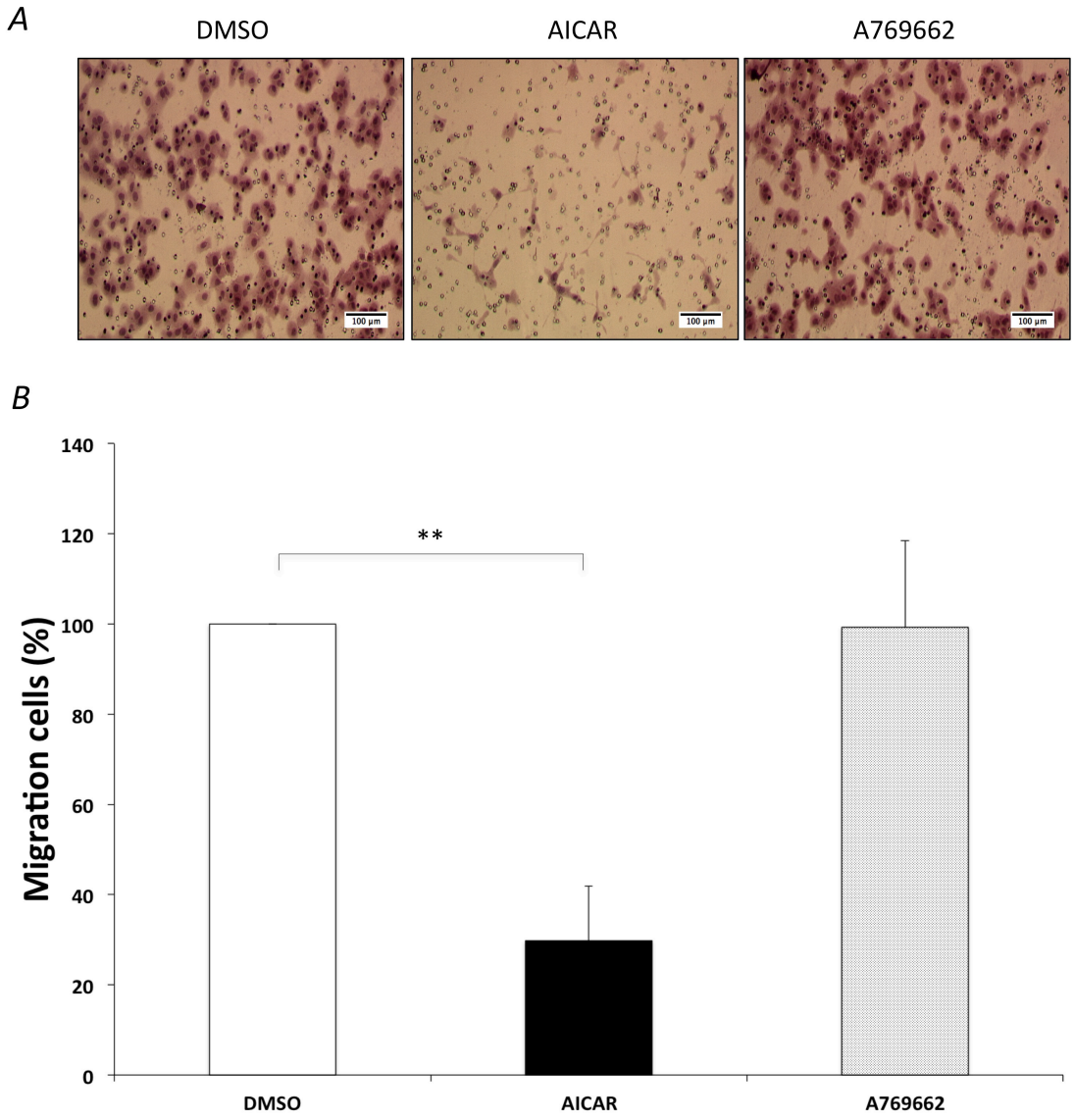


Figure 4.14 Effect of AMPK activators on DU145 cell chemotaxis

DU145 cells were incubated for 24 h in serum-free medium prior to being seeded in 8 μm pore size transwell inserts in serum-free medium. AICAR (1 mM) and A769662 (100 μM) were added in both the transwell inserts and 24-well chamber and incubated for 21 h. The inserts were then fixed with methanol and stained with Haemotaxylin. Four fields were analysed for each replicate. Scale bar represents 100 μm. (A) Representative images. (B) Quantification of migrated cells (**: $p<0.01$, N=6).

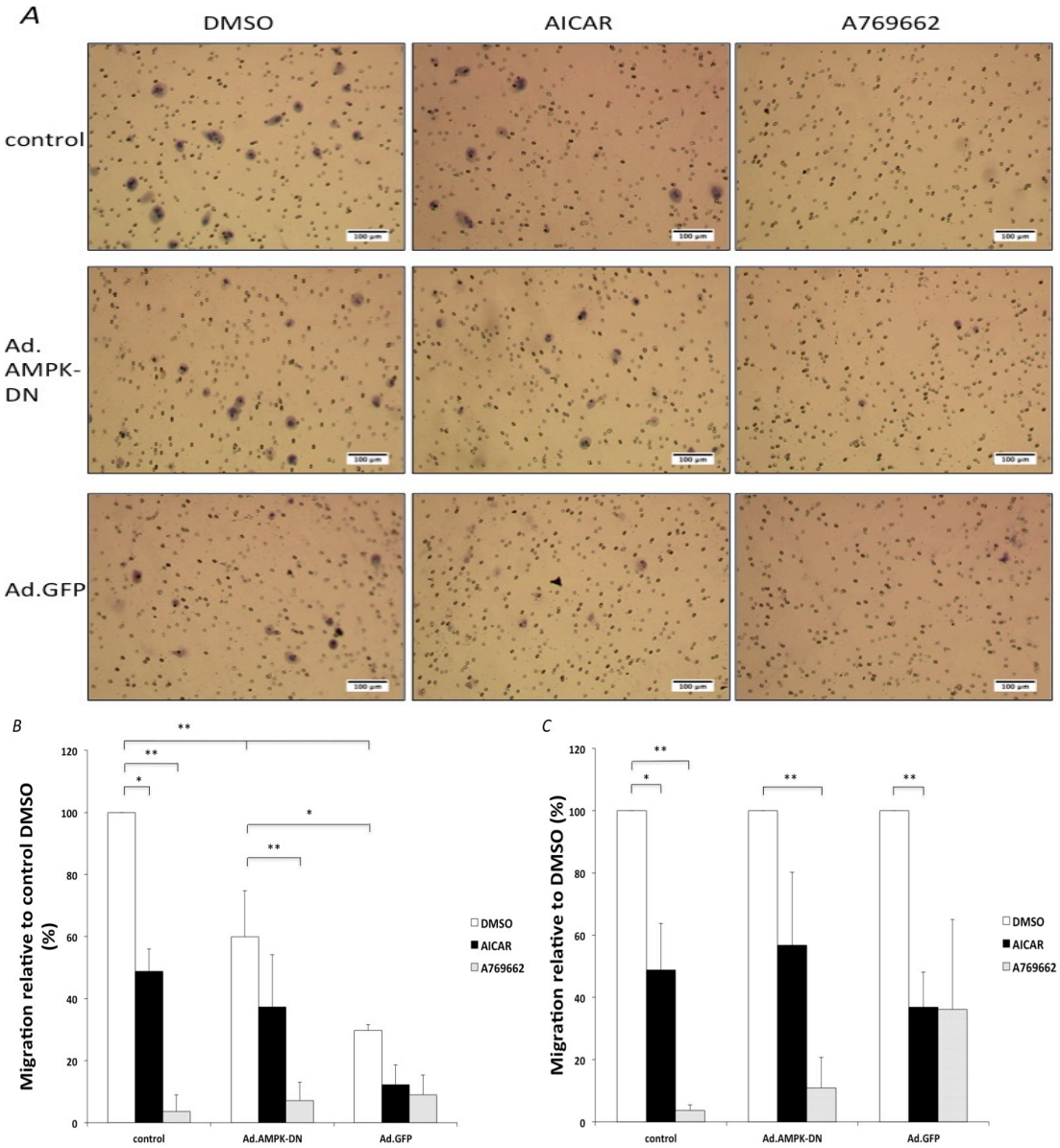


Figure 4.15 The effect of infection with Ad.AMPK-DN on inhibition of migration by AICAR and A769662 in PC3 cells

PC3 cells were infected with Ad.AMPK-DN or Ad.GFP at 200 IFU/cell and incubated for 24 h in full medium prior to being seeded in 8 μm pore size transwell inserts in serum-free medium. AICAR (1 mM) and A769662 (100 μM) were added in both transwell inserts and the underlying chambers and incubated for 21 h. The inserts were then fixed with methanol and the underside stained with Haemotaxylin. Four fields were analysed for each replicate. Scale bar represents 100 μm. (A) Representative images, (B) Migrated cells normalised to vehicle control of uninfected cells (*: $p<0.05$, **: $p<0.01$, N=3). (C) Migrated cells with in-group normalisation to DMSO control (*: $p<0.05$, **: $p<0.01$, N=3).

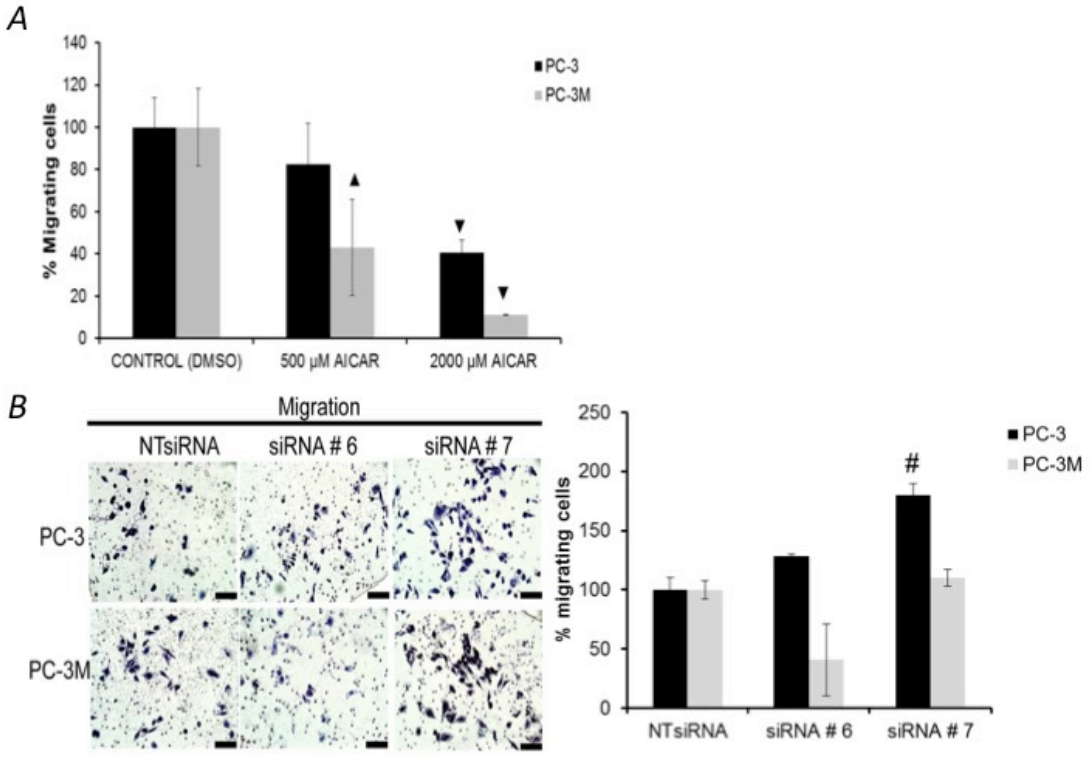


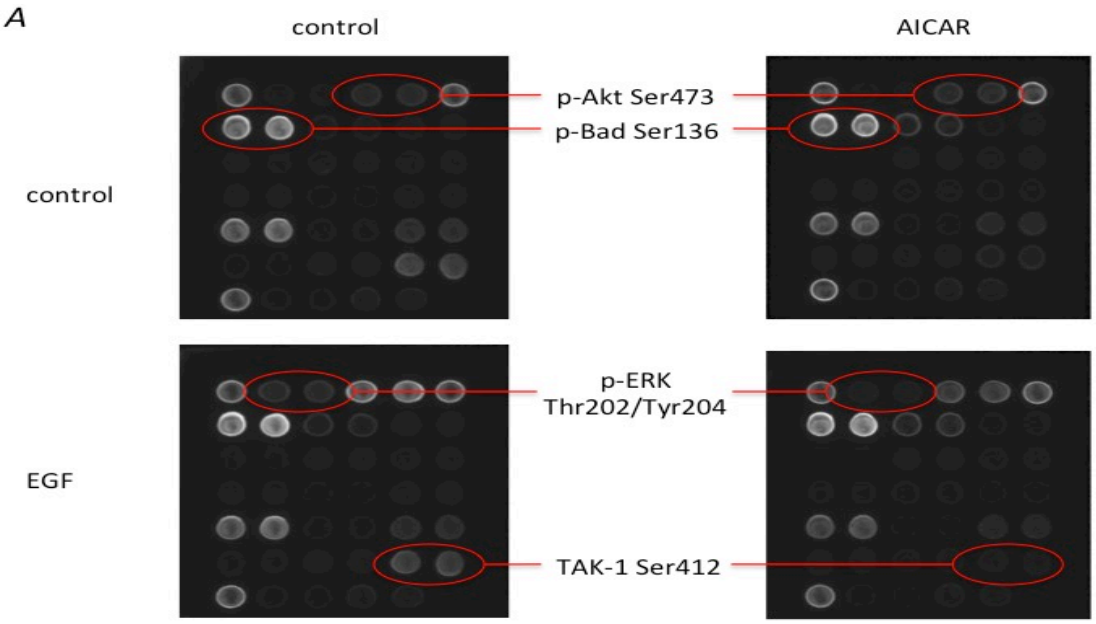
Figure 4.16 The effect of AICAR on cell migration in PC3 and PC3M cells

(A) Transwell assay for migration in the presence of AICAR (500 and 2000 μM). (B) Transwell assay after incubation of *PRKAA1* siRNA over 21 h, scale bar represents 100 μm. Data are presented as mean ± SEM of three independent experiments. #: p≤0.005, ▲: p≤0.01, ▼: p≤0.05 from (A: DMSO control, B: NT siRNA). These experiments were performed by Dr Yashmin Choudhury (University of Glasgow).

This figure is reproduced under the Creative Commons Attribution License using Figure 4C and 4E from article "AMP-activated protein kinase (AMPK) as a potential therapeutic target independent of PI3K/Akt signaling in prostate cancer" by Choudhury et al, Oncoscience (2014); 1(6) 446-456.

4.2.4 The effect of AMPK activators on proliferation and apoptosis signalling proteins in PC3 cells

To examine whether AICAR or A769662 influenced particular proliferation and/or apoptosis signalling proteins, PC3 cell lysates were analysed using a cell apoptosis signalling array, which is a pre-prepared immunoblotting panel containing different proteins of interest. Bcl-2-associated death promoter (BAD), a pro-apoptotic factor, which can be inhibited by phosphorylated at Ser136 by Akt, is used as normalisation control, due to its robust and consistent signal in these experiments. Both AICAR and A769662 caused a marked reduction in epidermal growth factor (EGF)-stimulated phospho-ERK1/2 and phospho-Akt levels (Ser473). In addition, AICAR, but not A769662 inhibited basal and EGF-stimulated TAK-1 (Ser412) phosphorylation. (Figure 4.17) This phenomenon is particularly interesting in the context of AMPK activation, as TAK-1 has been claimed to be an AMPK upstream kinases. Note – Anti-phospho ERK1/2 antibodies recognise both species with equal affinity as the epitope is completely conserved between ERK1 and ERK2.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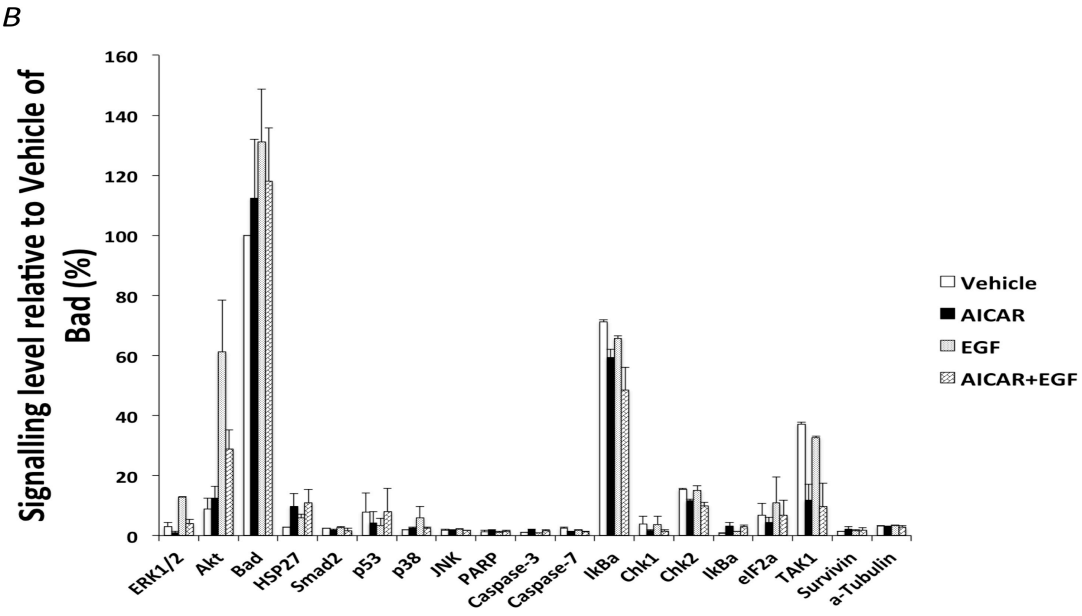
control
AICAR
control
p-Akt Ser473
p-Bad Ser136
EGF
p-ERK
Thr202/Tyr204
TAK-1 Ser412


B
Signalling level relative to Vehicle of Bad (%)
160
140
120
100
80
60
40
20
0
ERK1/2
Akt
Bad
HSP27
Smad2
p53
p38
JNK
PARP
Caspase-3
Caspase-7
IkBa
Chk1
Chk2
IkBa
eIF2a
TAK1
Survivin
a-Tubulin
Vehicle
AICAR
EGF
AICAR+EGF

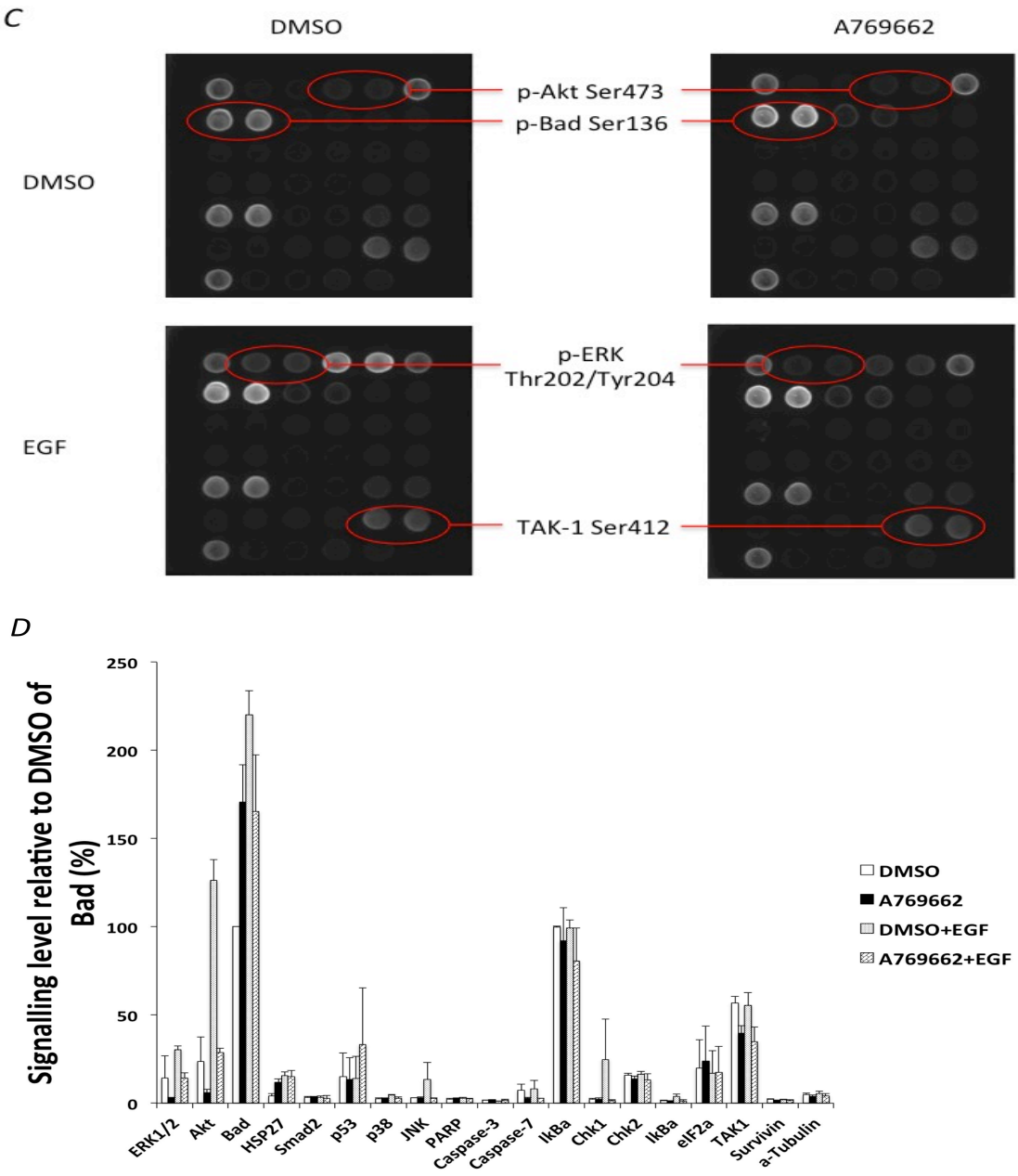


Figure 4.17 Apoptosis signalling array in PC3 cells after 72 h incubation with AICAR or A769662

PC3 cells were incubated for 2 h in serum-free medium before incubation with AICAR (1 mM) or A769662 (100 μM) for 72 h. EGF (10 ng/mL) was subsequently added for 15 min and lysates prepared. Protein lysates were analysed using a PathScan Apoptosis Assay according to the manufacturer's protocol. (A) Representative images of apoptosis signalling array. (B) Densitometric analysis for AICAR-stimulated cell lysates compared to vehicle (N=2). (C) Representative images of apoptosis signalling array. (D) Densitometric analysis for A769662-stimulated cell lysates compared to DMSO vehicle (N=2).

4.2.5 Effect of ERK and Akt inhibition on PC3 cell viability

Since the cell apoptosis signalling assay revealed that both AICAR and A769662 could decrease ERK1/2 and Akt phosphorylation in PC3 cells, the effect of ERK1/2 and Akt inhibition on PC3 cell viability was compared with the inhibitory effects of AICAR and A769662 using the MEK1/2 inhibitor PD184352 and Akt inhibitor Akti. Both PD184352 and Akti inhibited PC3 cell viability, an effect that was more marked when PD184352 and Akti were used in combination. Furthermore, the extent of inhibition was similar to that observed for AICAR. A769662 markedly reduced cell viability, which led to death of virtually all PC3 cells, such that lysates prepared contained no protein, therefore no statistical analysis was performed. (Figure 4.18) Akt phosphorylation at Ser473 and Thr308 was inhibited by Akti, but not by PD184352. ERK1/2 phosphorylation could not be detected in these unstimulated cells. Neither PD184352 nor Akti had any effect on ACC or AMPK phosphorylation (Figure 4.18).

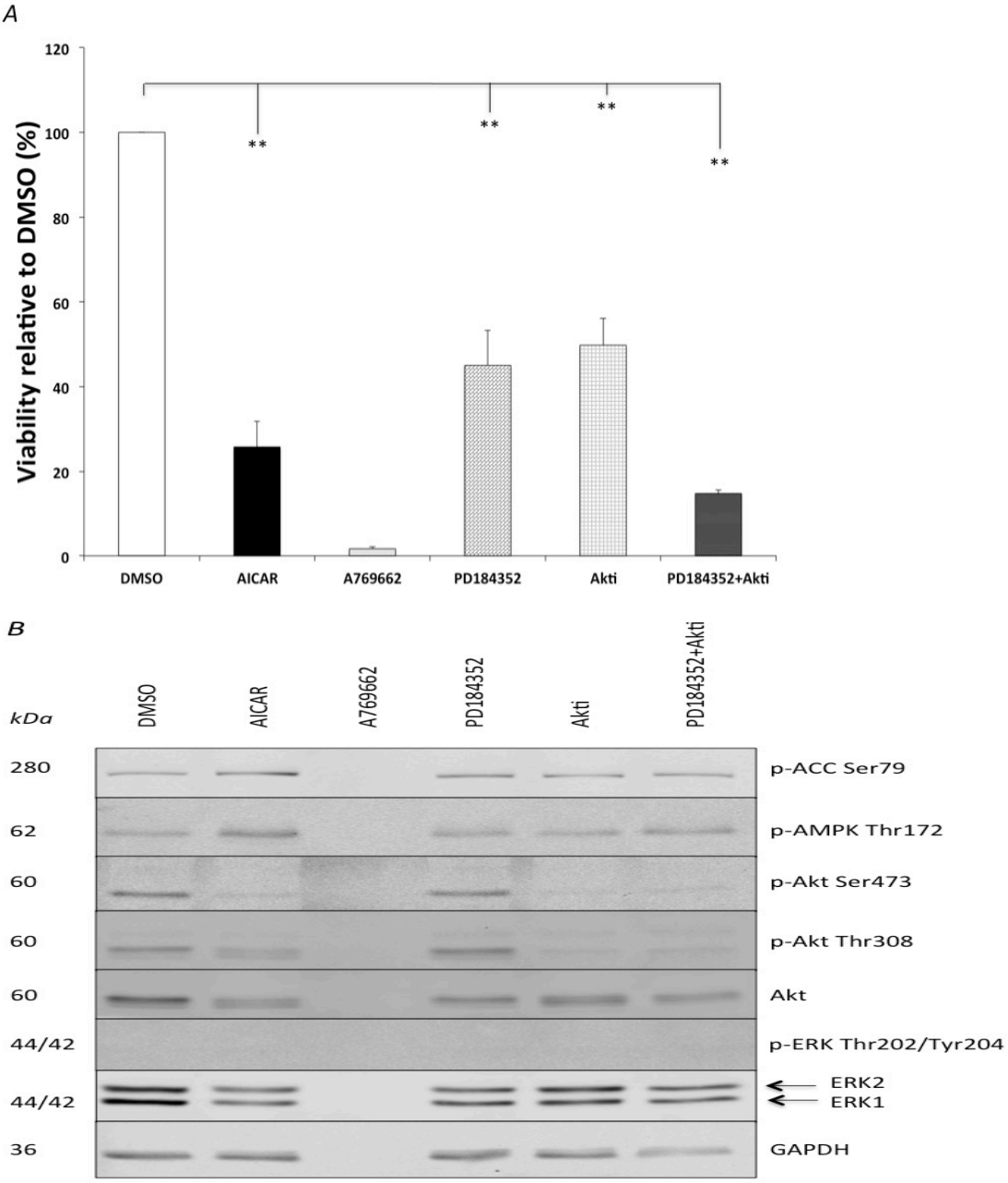


Figure 4.18 Viability assay of PC3 cells 72 h after incubation with AMPK activators, PD184352 and Akti

Cells were incubated for 2 h in serum-free medium before incubation in the presence or absence of the following compounds for 72 h: AICAR (1 mM), A769662 (100 μM), PD184352 (3 μM), Akti (1 μM). (A) WST-1 (10% (v/v)) was added to each well and normalised absorbance was measured after 120 min. Cell viability was normalised to DMSO control (**: $p<0.01$, N=3). (B) Protein lysates were prepared, resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated (N=1). GAPDH was used as loading control.

4.3 Discussion

The key findings of this study were that AICAR and A769662, which activate AMPK by distinct mechanisms, both inhibited proliferation and migration in human PC cell lines, an effect associated with reduced ERK1/2 and Akt phosphorylation. Firstly, effect of AMPK activation using AICAR and A769662 on PC cell viability was analysed using the WST-1 method. WST-1 is a long established colourimetric assay based on assessing the metabolic activity of the cells, which can then be used as an indication of cell proliferation (Slater *et al.*, 1963, Carmichael *et al.*, 1987, Cook and Mitchell, 1989). Incubation with either AICAR or A769662 for 72 h decreased cell viability in PC3 and DU145 cells. The degree of reduction is similar in PC3 cells for either AICAR or A769662, whereas AICAR seems to be more effective in DU145 cells. To examine whether the reduction in proliferation was due to AMPK activation, AMPK DN adenoviruses Ad.AMPK-DN were used to repeat the WST-1 experiments. Incubation with AICAR or A769962 for 72 h was still able to reduce cell viability in both PC3 and DU145 cells, and there was no obvious difference between Ad.AMPK-DN infected cells and Ad.GFP infected cells. These results indicate that the effect of reduced proliferation after incubation with AMPK activators may be AMPK independent. However, it can be argued that there may have been insufficient AMPK activity inhibition in those experiments. Therefore, WT and *AMPK* $\alpha 1^{-/-}$ *AMPK* $\alpha 2^{-/-}$ KO MEFs were used to carry out proliferation experiments using AICAR and A769662 by assessing BrdU incorporation, which is an accurate analysis of proliferation by measuring DNA synthesis (Plickert and Kroiher, 1988). Since there may be a different metabolic profile between WT and *AMPK* $\alpha 1^{-/-}$ *AMPK* $\alpha 2^{-/-}$ KO MEFs, it is also better to measure proliferation using the BrdU method instead of WST-1, as differences in metabolic status would not affect the results. Incubation with AICAR led to a concentration-dependent reduction in cell proliferation in both WT and *AMPK* $\alpha 1^{-/-}$ *AMPK* $\alpha 2^{-/-}$ KO MEFs, with more reduced proliferation in *AMPK* $\alpha 1^{-/-}$ *AMPK* $\alpha 2^{-/-}$ KO MEFs. These results suggest that AICAR induced reduction in proliferation is not due to AMPK activation. Incubation with A769662 however, reduced proliferation in WT MEFs at a concentration of 30 μM, and the inhibition of proliferation was significantly less in *AMPK* $\alpha 1^{-/-}$ *AMPK* $\alpha 2^{-/-}$

KO MEFs, suggesting that the effect of A769662 may be (at least partially) through AMPK activation. It is worth noting that higher concentrations of A769662 at 100 μM lead to cell death, so the great reduction in proliferation at that concentration is difficult to interpret.

Secondly, the effect of AMPK activation on PC cell migration was carried out using scratch wound assays (Valster *et al.*, 2005, Liang *et al.*, 2007), which provide useful information regarding cell mobility. In PC3 cells, both AICAR and A7696662 have a tendency for reduced cell mobility. In DU145 cells however, this tendency is less obvious when compared to PC3 cells. This might be due to the relatively low basal cell mobility in DU145 Cells. Cell tracking analyses indicate that AICAR and A769662 can reduce accumulated distance, Euclidean distance and velocity in PC3 and DU145 cells, although the significance of the results could not be assessed due to the small sample size. Using the transwell method in PC3 cells, both AICAR and A769662 decreased cell migration, however, the huge reduction in A769662 might be due to cell death. In DU145 cells, only AICAR can decrease cell migration as assessed using the transwell assay, but not A769662. The AMPK-dependence of this effect of the AMPK activators is unclear as the inhibitory effect of AICAR loses significance in PC3 cells infected with Ad.AMPK-DN, indicating that AMPK activation might partly contribute to cell migration.

Either AICAR or A769662 incubation was associated with decreased EGF-stimulated phospho-Akt Ser473 and p-ERK1/2 (Thr202/Tyr204). AICAR could also decrease TAK-1 (Ser412) phosphorylation, although A769662 had no effect. Incubation of PC3 cells with the ERK inhibitor PD184352 and Akt inhibitor Akti alone or in combination inhibited PC3 cell proliferation and combined incubation of PD184352 and Akti produced a similar extent of inhibition to AICAR in cell viability.

Since the start of this project, several studies have also shown that AMPK activation could inhibit cell proliferation in PC cell lines. Metformin has been reported to decrease PC3, DU145 and LNCaP cell viability as assessed by cell

counting (Tsutsumi *et al.*, 2015). Furthermore, a recent study reported that plectranthoic acid, an AMPK activator, can decrease PC3 and DU145 cell proliferation as determined by MTT assay (Akhtar *et al.*, 2016). Another group also demonstrated that both AICAR and MT 63-78, a molecule which directly activates AMPK, inhibit cell proliferation measured by cell counting in PC3, LNCaP and other PC cell lines (Zadra *et al.*, 2014). Before the start of this project, there was no evidence showing the effect of AMPK activators on PC cell motility, although others had reported that overexpression of CaMKK2 or adiponectin, which was associated with increased AMPK phosphorylation, led to enhanced cell migration (Tang and Lu, 2009, Frigo *et al.*, 2011). In contrast, the data presented in this chapter indicates that AMPK activators decrease migration and inactivation of AMPK by *PRKAA1* siRNA increased cell migration. These data support a similar previous finding in C4-2 cells (Zhou *et al.*, 2009). Also, a recent study has reported that metformin can decrease cell viability but not mobility in PC3, DU145 and LNCaP cells (Tsutsumi *et al.*, 2015). It was demonstrated in the above experiments that A769662 had marked effects on cell viability and migration comparing to AICAR. Although these effects might be explained by the off-target A769662 toxicity (Moreno *et al.*, 2008), interestingly, in DU145 cells, theses effects were far less sensitive comparing with PC3 cells. Giving the fact that DU145 cells lack LKB1, one of AMPK upstream kinases, it is even more interesting as the effects of A769662 should not be affected by LKB1 status.

In conclusion, this chapter studied the functional effects of two AMPK activators AICAR and A769662, on PC cell including proliferation and migration. The results suggest that both AICAR and A769662 have potential anti-tumourigenic properties by suppressing cell proliferation and migration. The anti-proliferative and anti-migratory effects of AICAR and A769662 may be AMPK-independent, although compound C and siRNA targeted against AMPK α1 do partially reverse the effects of A769662 and AICAR on proliferation in PC3 cells and A769662 in MEFs. Moreover, using an apoptosis array, AMPK activators suppressed ERK1/2 and Akt signalling and the effects of ERK1/2 inhibition and Akt inhibition mimicked the effects of AICAR, suggesting the potential anti-tumourigenic actions of AMPK activators may be mediated by the MAPK and PI3K/Akt pathways.

Chapter 5. The effects of AMPK activation on epidermal growth factor (EGF)-stimulated mitogen-activated protein kinase (MAPK) signalling pathways in human prostate cancer cell lines

5.1 Introduction

5.1.1 Brief overview of MAPK pathway

MAPK pathways are serine/threonine protein kinases that regulate many cell functions and respond to multiple signals including growth factors and extracellular stress (Pearson *et al.*, 2001, Turjanski *et al.*, 2007). The signalling cascade is a three-level module system. Upon activation, the signalling pathway activates an MAPKKK, which phosphorylates and activates an MAPKK, which then phosphorylates and activates an MAPK (Pearson *et al.*, 2001, Dhillon *et al.*, 2007).

5.1.2 Grouping and function

At least 11 members of the MAPK superfamily have been observed in humans, which can be characterised into seven groups as follows (Schaeffer and Weber, 1999, Pearson *et al.*, 2001, Klinger and Meloche, 2012, Morrison, 2012, Arthur and Ley, 2013, Yang *et al.*, 2013):

1) ERK1/2
2) JNK (JNK1, JNK2, JNK3)
3) p38 (p38α, p38β, p38γ, p38δ)
4) ERK3/4
5) ERK5
6) Nemo like kinase
7) ERK7

Individual MAPKs have distinct physiological functions (Seger and Krebs, 1995). For instance, ERK1/2, the best-characterised pathway, regulates a wide range of functions including cell proliferation, differentiation, apoptosis, migration and cell cycle control (Seger and Krebs, 1995, Dhillon *et al.*, 2007). JNK and p38 pathways, in the other hand, are often activated by cytokines and cellular stresses (Weston and Davis, 2002, Dhillon *et al.*, 2007), but are similarly involved in cell proliferation, differentiation and apoptosis (Dhillon *et al.*, 2007).

5.1.3 Abnormal MAPK signalling in prostate carcinogenesis

Abnormal MAPK signalling pathways have been found to have a significant contribution in tumourigenesis as well as disease progression (Dhillon *et al.*, 2007). Evidence has shown that expression and activation of the MAPKs including ERK1/2, JNK and p38 can be detected in prostate cancer (PC) tissue (Uzgare *et al.*, 2003). Studies also suggest that p38 activation in androgen-independent AR activity may contribute to an aggressive androgen-independent phenotype in PC (Khandrika *et al.*, 2009). The role of JNK in PC, on the other hand, is poorly understood, and JNK has been suggested to promote or suppress oncogenesis in different settings (Manning and Davis, 2003). ERK5, one of the four identified ERK pathways in mammals, is a unique pathway in terms of the distinct molecular mass of ERK5, its activity and role (Dhillon *et al.*, 2007, Turjanski *et al.*, 2007, Yang *et al.*, 2010). ERK5 contributes to carcinogenesis by promoting proliferation, migration, invasion and angiogenesis (Lochhead *et al.*, 2012). In PC, this is due to the promotion of cellular motility and invasion, rather than increased proliferation (Ramsay *et al.*, 2011). In addition, ERK5 mediated neo-angiogenesis is also required for carcinogenesis *in vivo* (Hayashi *et al.*, 2005).

5.1.4 Therapeutic potential of MAPK inhibition

The first MEK1/2 inhibitor, PD98059, was discovered in 1995 (Dudley *et al.*, 1995), yet it was not approved for clinical use (Fremin and Meloche, 2010). Fifteen years later, eleven MEK1/2 inhibitors targeting the ERK1/2 pathways had been tested clinically or were being tested in clinical trials (Fremin and Meloche, 2010). Recent studies have also shown that MEK inhibitors have merit in the treatment of aggressive PC *in vivo* (Ahmad *et al.*, 2011). In addition to anti-MEK1/2 agents, other MAPK inhibitors targeting p38, JNK or RAF have also been developed as therapies in malignancy as well as in cardiovascular and inflammatory diseases (Roberts and Der, 2007). Combination of inhibitors of other MAPK pathways together with MEK1/2 inhibitors has been reported to be more effective than each one alone in different types of cancer (Meng *et al.*, 2010, Naderi *et al.*, 2011, Tanizaki *et al.*, 2012, Zhao *et al.*, 2012). Inhibition of ERK1/2 and p38 pathways, for instance, either alone or together, could provide inhibition

of human PC invasion and metastasis (Chen *et al.*, 2004). Paradoxically, Gan *et al* suggested that pharmacological inhibition of the ERK pathway could enhance EGF-induced EGFR activation (Gan *et al.*, 2010). Researchers have shown that hypoxia-reoxygenation can lead to enhanced survival and invasiveness in LNCaP cells, and the hypoxia-reoxygenation is associated with increased AR activity independent of androgens (Khandrika *et al.*, 2009). Inhibition of p38 could eliminate this hypoxia-reoxygenation induced AR activity and its associated increased survival and invasiveness (Khandrika *et al.*, 2009). In contrast, only a few JNK inhibitors are being considered as a treatment of cancer (Manning and Davis, 2003, Roberts and Der, 2007). Down-regulation of ERK5 has also been shown to have beneficial effects for both hepatocellular carcinoma and PC *in vitro* and *in vivo* (Zen *et al.*, 2009, Ramsay *et al.*, 2011). Thus, ERK5 is a potential therapeutic target for PC. Indeed, inhibition of this pathway by XMD8-92 (a specific ERK5 inhibitor) can reduce tumour cell proliferation in both A549 and HeLa, which in turn inhibits tumour growth (Yang *et al.*, 2010).

5.1.5 EGF as a stimulus of MAPK pathway

Growth factors are a group of polypeptides and proteins playing an important part in many aspects of physiology (Barrett *et al.*, 2016). Biologically, EGF exerts its function by binding to the EGF receptor (EGFR) (Herbst, 2004). It has been recognised that EGFR abnormality is closely associated with cancer (Lynch *et al.*, 2004, Normanno *et al.*, 2006). This has led to research concerning the potential of manipulating EGFR as a therapeutic target in cancer (Mendelsohn and Baselga, 2000). It has been established for more than two decades that EGF could trigger the MAPK signalling cascades (Ahn and Krebs, 1990, Anderson *et al.*, 1990, Ahn *et al.*, 1991, Seger and Krebs, 1995). As shown in Chapter 4, AICAR- and A769662-mediated inhibition of proliferation and migration were associated with reduced EGF-stimulated ERK1/2 phosphorylation. The current study therefore further examined the rapid and long-term effects of AMPK activators on MAPK signalling pathways in PC cell lines.

5.2 Results

5.2.1 Effects of AMPK activation on EGF-stimulated MAPK signalling in prostate cancer cells

5.2.1.1 Rapid and long-term effects of AMPK activators on ERK1/2 phosphorylation in prostate cancer cell lines

Experiments in Chapter 4 suggest that AMPK activators suppressed EGF-stimulated ERK1/2 phosphorylation in PC cell lines as assessed using a signalling immunoblotting array. The effect of rapid and long-term incubation with AMPK activators on ERK1/2 phosphorylation was therefore assessed by immunoblotting in various PC cell lines. EGF stimulated ERK1/2 phosphorylation in all three cell lines, and had no effect on AMPK activity as assessed by phospho-ACC Ser79 levels (Figures 5.1 to 5.4).

In PC3 cells, long-term (72 h) incubation (the same incubation time used in the proliferation experiments described in Chapter 4) with AICAR slightly decreased EGF-stimulated ERK1 but not ERK2 phosphorylation, although this did not reach statistical significance. A769662 on the other hand, significantly decreased EGF-stimulated ERK1 but not ERK2 phosphorylation (Figure 5.1). Short-term incubation with concentrations and durations of AICAR (135 min) or A769662 (75 min) determined to achieve maximal AMPK activation (Chapter 3) had no effect on basal or EGF-stimulated ERK1/2 phosphorylation in PC3 cells (Figure 5.2). In DU145 cells, short-term incubation with AICAR (135 min) or A769662 (45 min) (optimal time and concentration for AMPK activation, Chapter 3) had no effect on basal or EGF-stimulated ERK1/2 phosphorylation. However, A769662 robustly stimulated basal ERK1/2 phosphorylation (Figure 5.3), without further increasing EGF-stimulated ERK1/2 phosphorylation. In LNCaP cells, short-term incubation with AICAR (75 min) or A769662 (75 min) (optimal time and concentration for AMPK activation, Chapter 3) had no effect on basal or EGF-stimulated ERK1/2 phosphorylation (Figure 5.4). To determine whether the

effect of A769662 on basal ERK1/2 phosphorylation in DU145 cells is AMPK dependent, experiments were carried out using compound C, an AMPK inhibitor. Preincubation of DU145 cells with compound C had no effect on basal or A769662-stimulated ERK1/2 phosphorylation, although it clearly reduced A769662-stimulated AMPK activity, as assessed by phospho-ACC Ser79 (Figure 5.5).

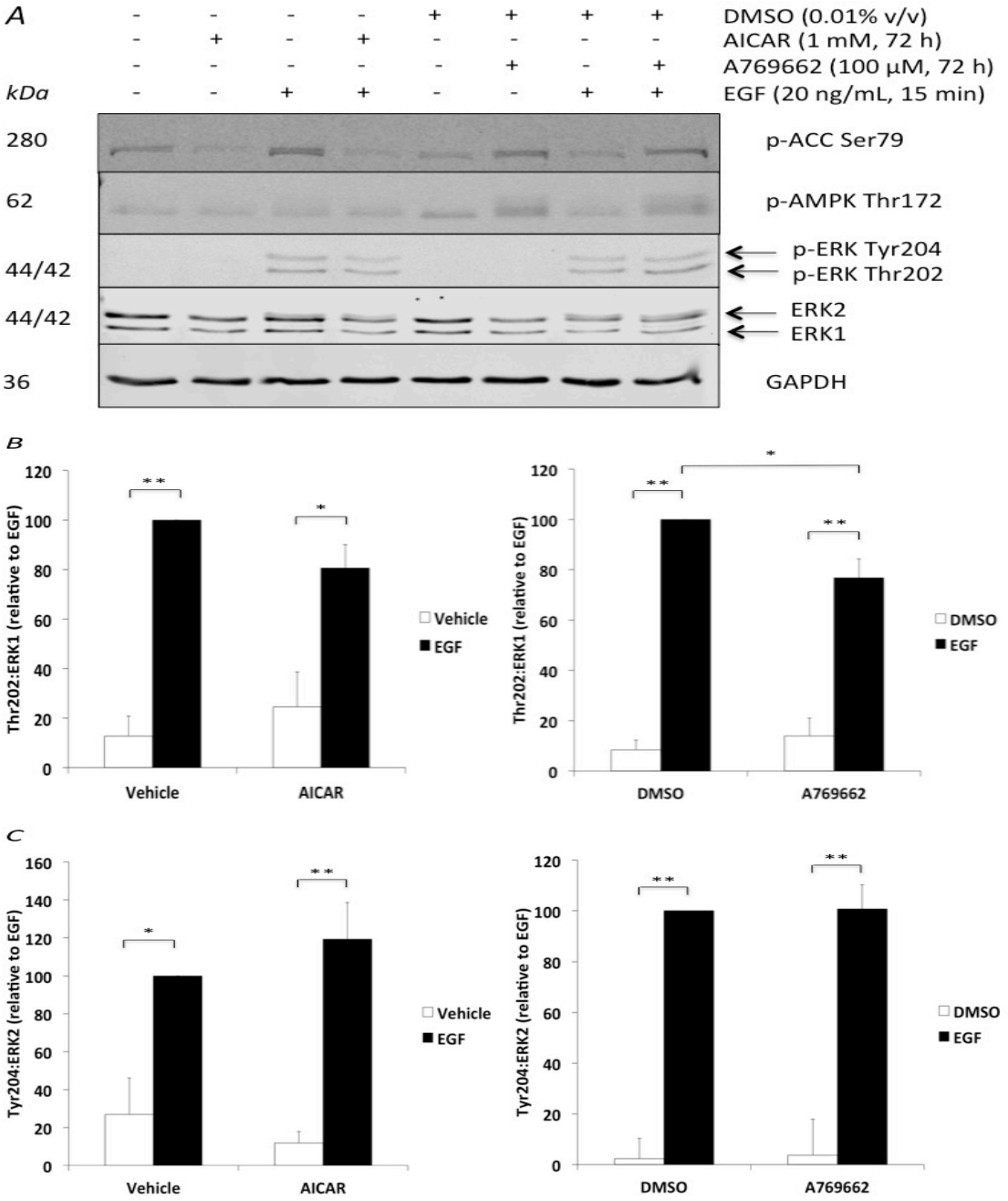


Figure 5.1 Long-term effect of AMPK activators on EGF-stimulated ERK1/2 phosphorylation in PC3 cells

PC3 cells were incubated for 2 h in serum-free medium before incubation with 1 mM AICAR or 100 μM A769662 for 72 h. EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of ERK1 phosphorylation level. (C) Densitometric analysis of ERK2 phosphorylation level (*: $p<0.05$, **: $p<0.01$ relative to absence of EGF, N=3).

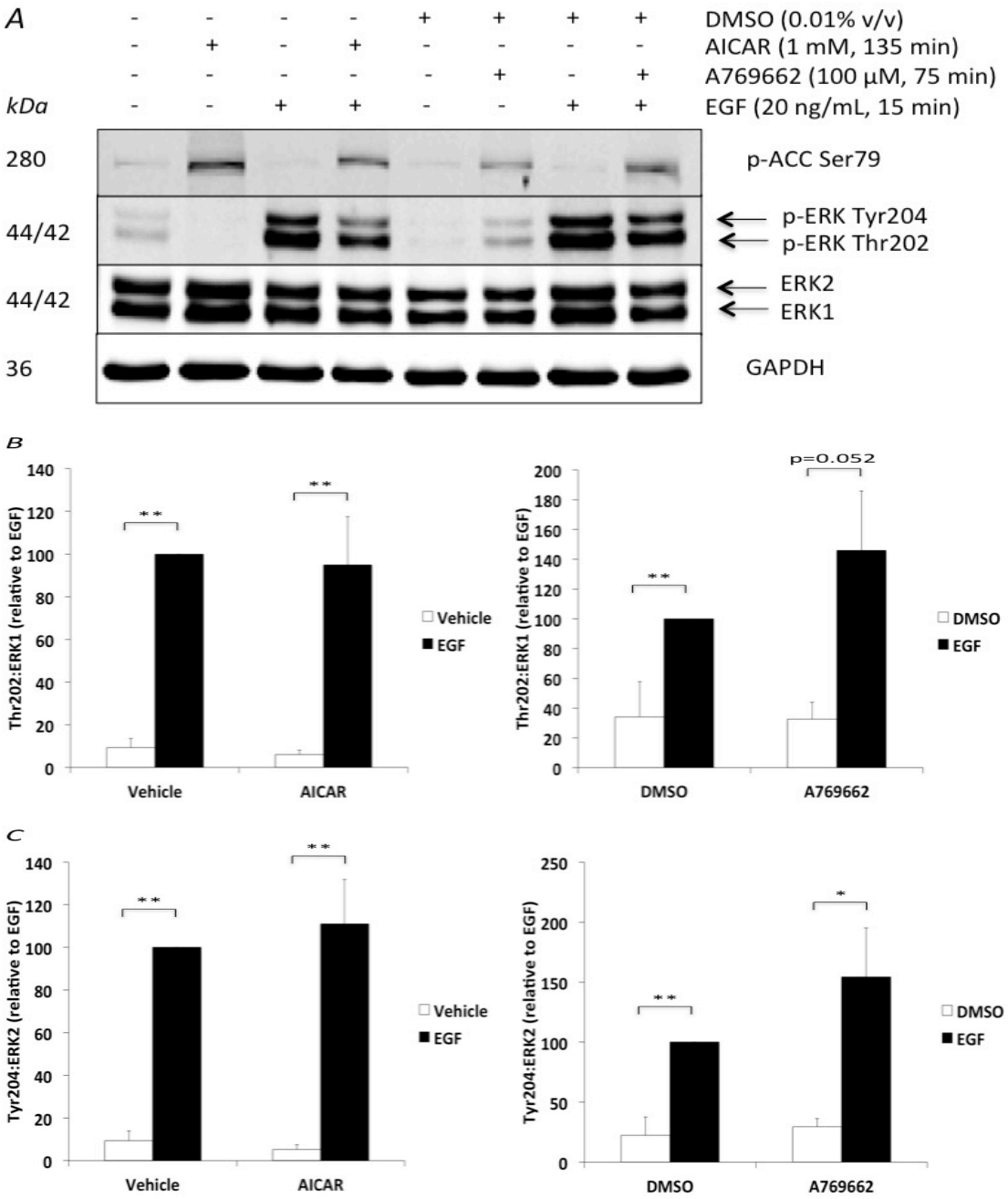


Figure 5.2 Effect of short-term incubation with AMPK activators on ERK1/2 phosphorylation in PC3 cells

PC3 cells were incubated for 2 h in serum-free medium before incubation with 1 mM AICAR (135 min) or 100 µM A769662 (75 min). EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of ERK1 phosphorylation level. (C) Densitometric analysis of ERK2 phosphorylation level (*: $p<0.05$, **: $p<0.01$ relative to absence of EGF, N=3).

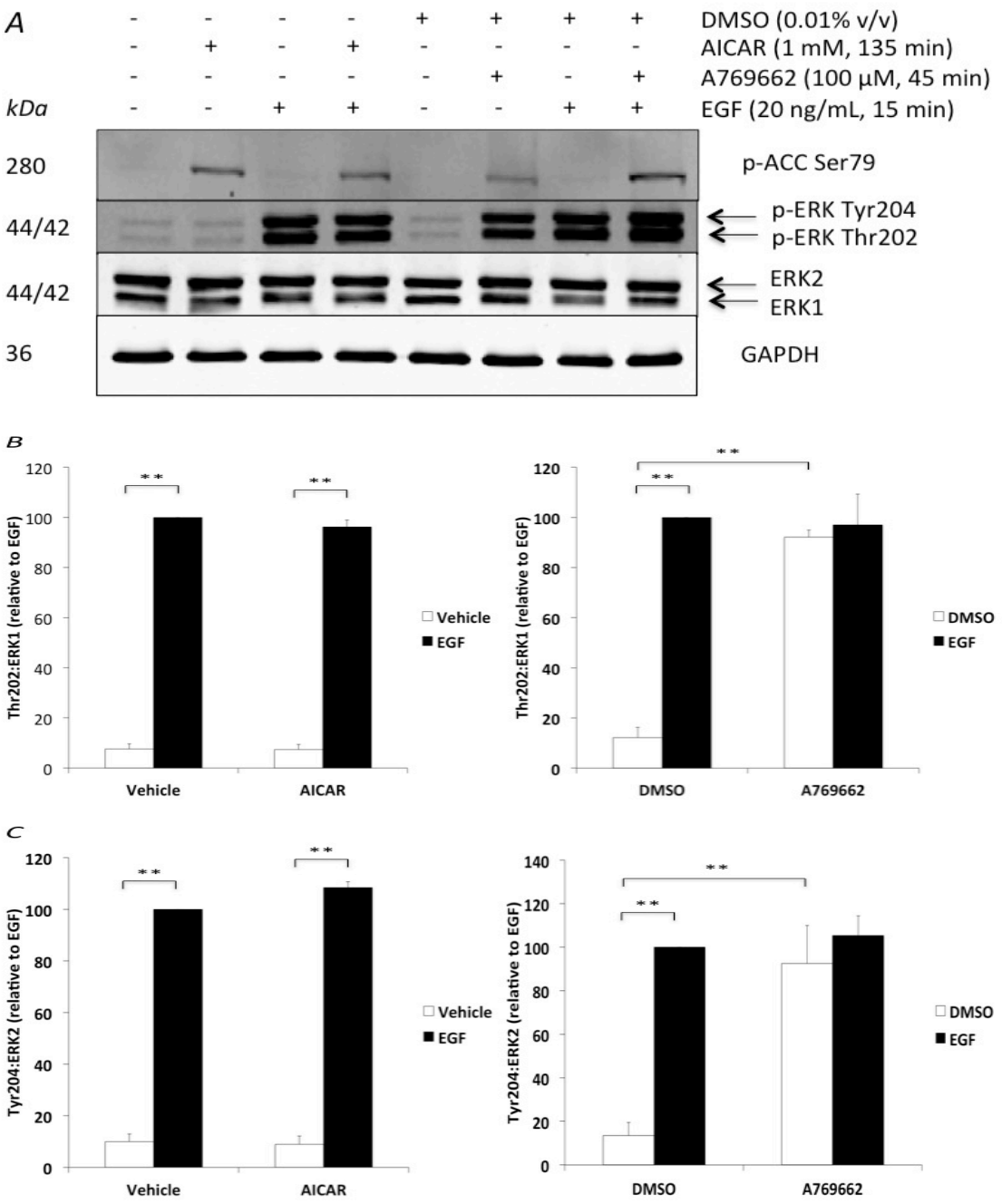


Figure 5.3 Effect of short-term incubation with AMPK activators on ERK1/2 phosphorylation in DU145 cells

DU145 cells were subjected to incubation for 2 h in serum-free medium before incubation with 1 mM AICAR (135 min) or 100 μM A769662 (45 min). EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of ERK1 phosphorylation level. (C) Densitometric analysis of ERK2 phosphorylation level (**: $p<0.01$, N=3).

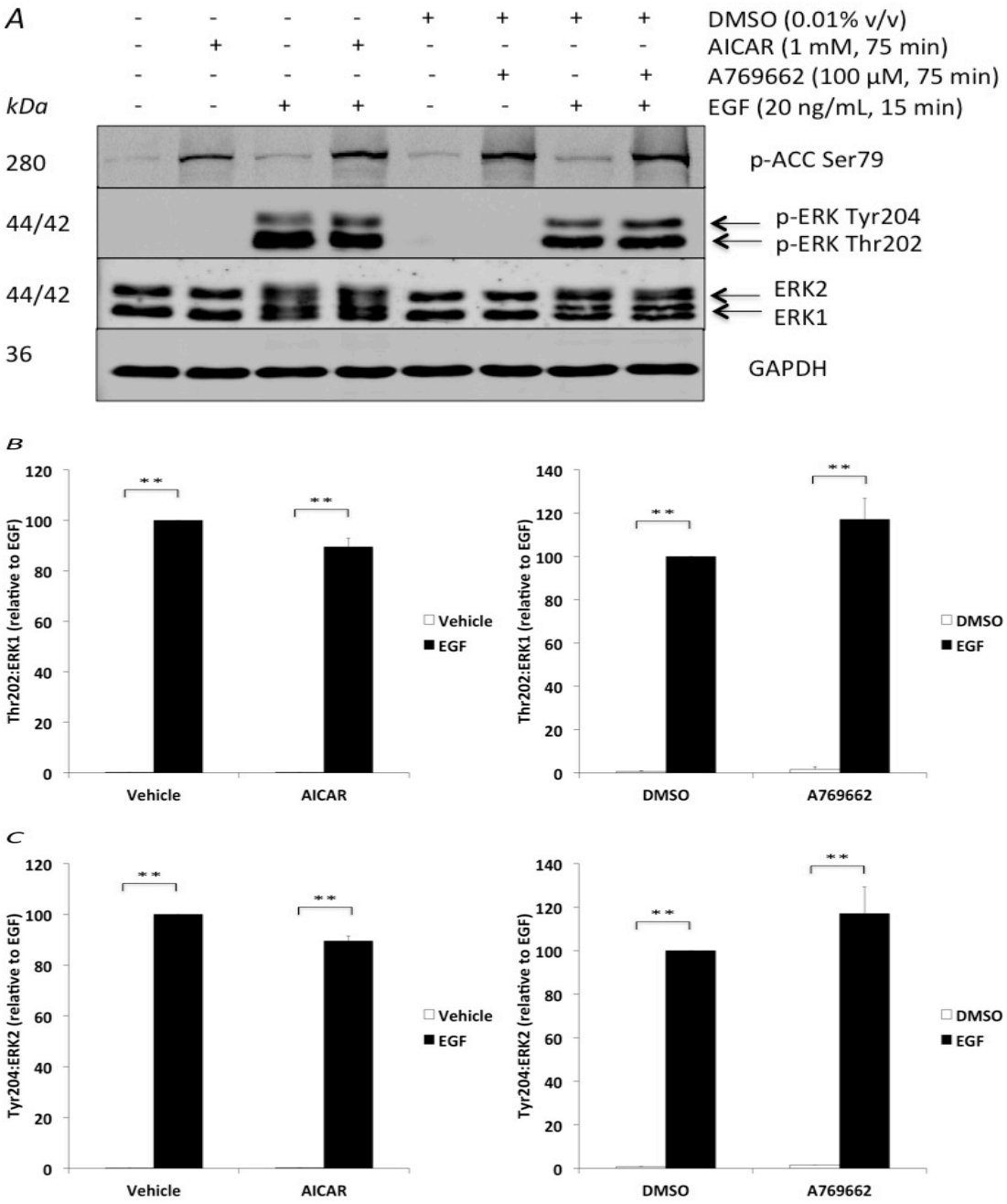


Figure 5.4 Effect of short-term incubation with AMPK activators on ERK1/2 phosphorylation in LNCaP cells

LNCaP cells were subjected to incubation for 2 h in serum-free medium before incubation with 1 mM AICAR (75 min) or 100 μM A769662 (75 min). EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of ERK1 phosphorylation level. (C) Densitometric analysis of ERK2 phosphorylation level (**: $p<0.01$ relative to absence of EGF, N=3).

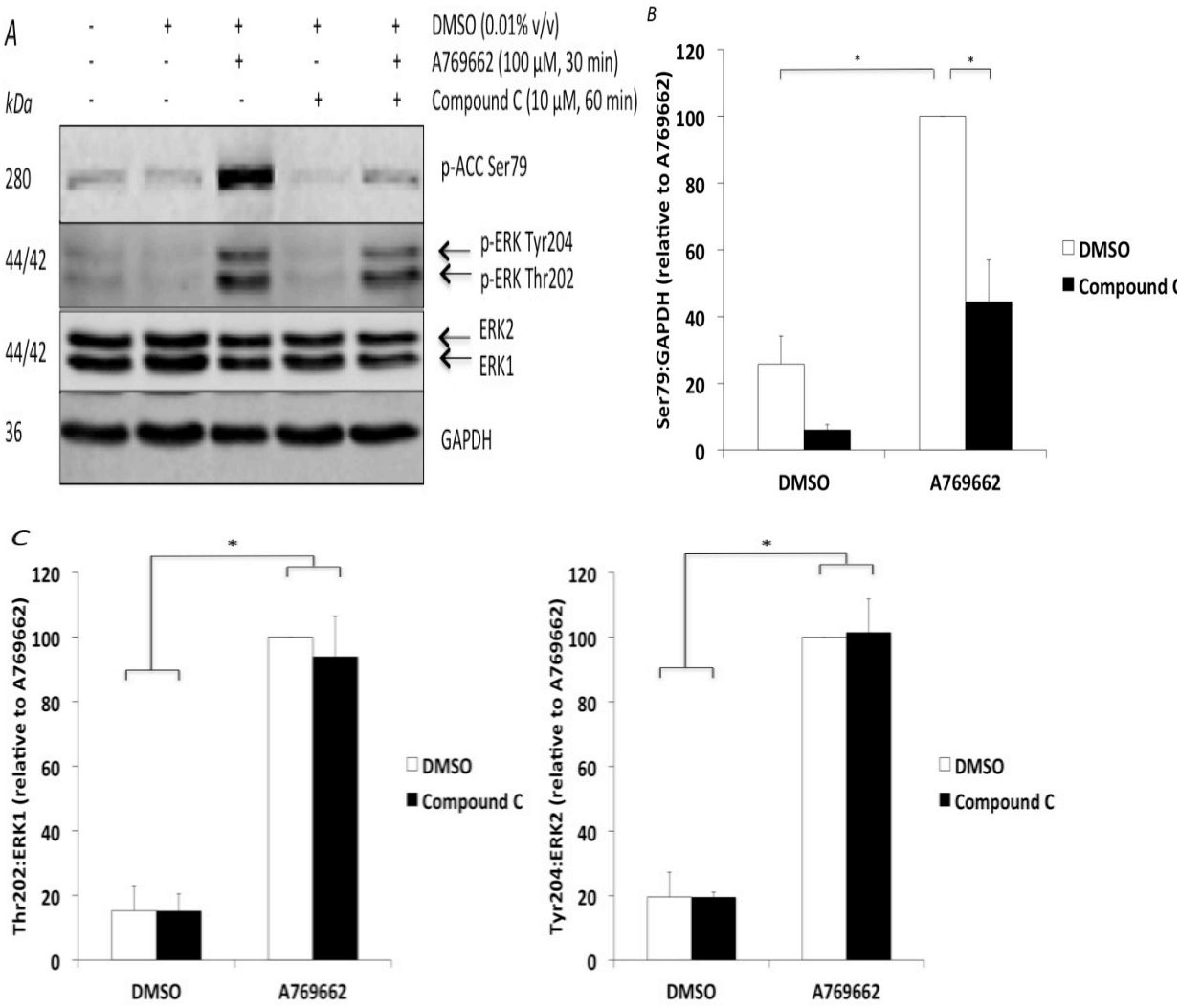


Figure 5.5 A769662-stimulated ERK1/2 phosphorylation in DU145 cells is unaffected by the AMPK inhibitor, compound C

DU145 cells were subjected to incubation for 2 h in serum-free medium before incubation with 10 μM compound C (60 min) prior to 100 μM A769662 (30 min). Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of ACC phosphorylation (*: $p<0.05$, N=3). (C) Densitometric analysis of ERK1 and ERK2 phosphorylation (*: $p<0.05$ ANOVA, N=3).

5.2.1.2 Effect of AMPK activators on JNK phosphorylation in prostate cancer cell lines

For all three PC cell lines used, EGF did not stimulate significant phosphorylation of JNK. In DU145, there is a tendency toward increased JNK phosphorylation after AICAR incubation (Figure 5.6). Apart from that, neither AICAR nor A769662 had any effect on JNK phosphorylation in the presence or absence of EGF (Figure 5.6).

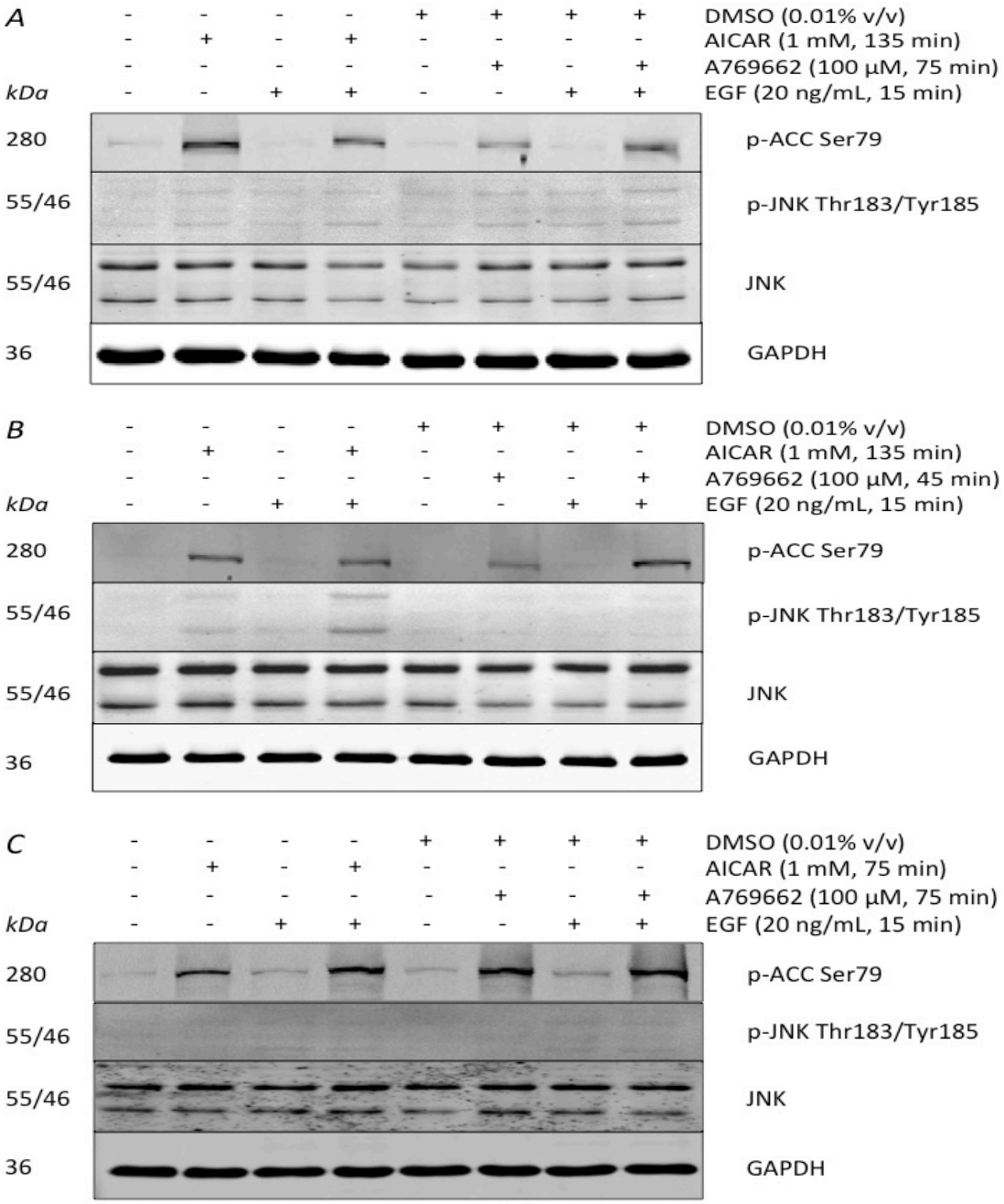


Figure 5.6 Effect of short-term incubation with AMPK activators on JNK phosphorylation in prostate cancer cell lines

(A) PC3 cells (B) DU145 cells or (C) LNCaP cells were incubated for 2 h in serum-free medium before incubation with 1 mM AICAR (135 min for PC3 and DU145 cells, 75 min for LNCaP cells) or 100 μM A769662 (75 min for PC3 and LNCaP cells, 45 min for DU145 cells). EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. Experiments were repeated at least three times with representative blots shown.

5.2.1.3 Effect of AMPK activators on p38 phosphorylation in prostate cancer cell lines

EGF stimulated p38 phosphorylation in all three cell lines. However, it only reached significance in DU145 and LNCaP cells (Figures 5.7, 5.8 and 5.9). In PC3 and DU145 cells, AICAR causes a modest, but not statistically significant increase in basal and EGF-stimulated p38 phosphorylation (Figures 5.7 and 5.8). In DU145 cells, A769662 also increased basal phosphorylation of p38, yet this did not reach significance. In addition, A769662 modestly, though statistically insignificant, inhibited EGF-stimulated p38 phosphorylation in DU145 (Figure 5.8). In PC3 and LNCaP cells, neither AICAR nor A769662 had any effect on EGF-stimulated p38 phosphorylation (Figures 5.7 and 5.9).

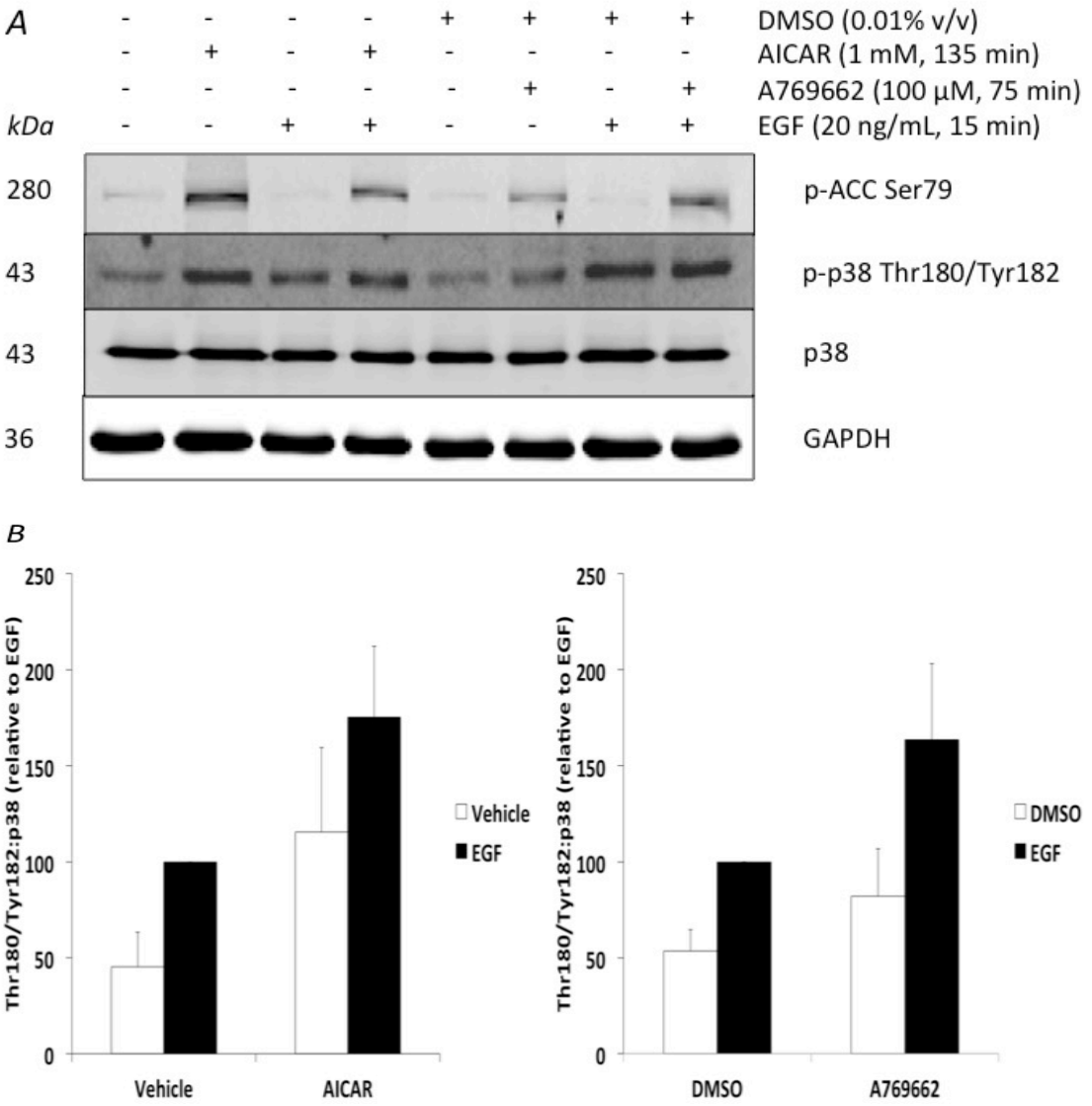


Figure 5.7 Effect of short-term incubation with AMPK activators on p38 phosphorylation in PC3 cells

PC3 cells were incubated for 2 h in serum-free medium before incubation with 1 mM AICAR (135 min) or 100 µM A769662 (75 min). EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of p38 phosphorylation level (N=3).

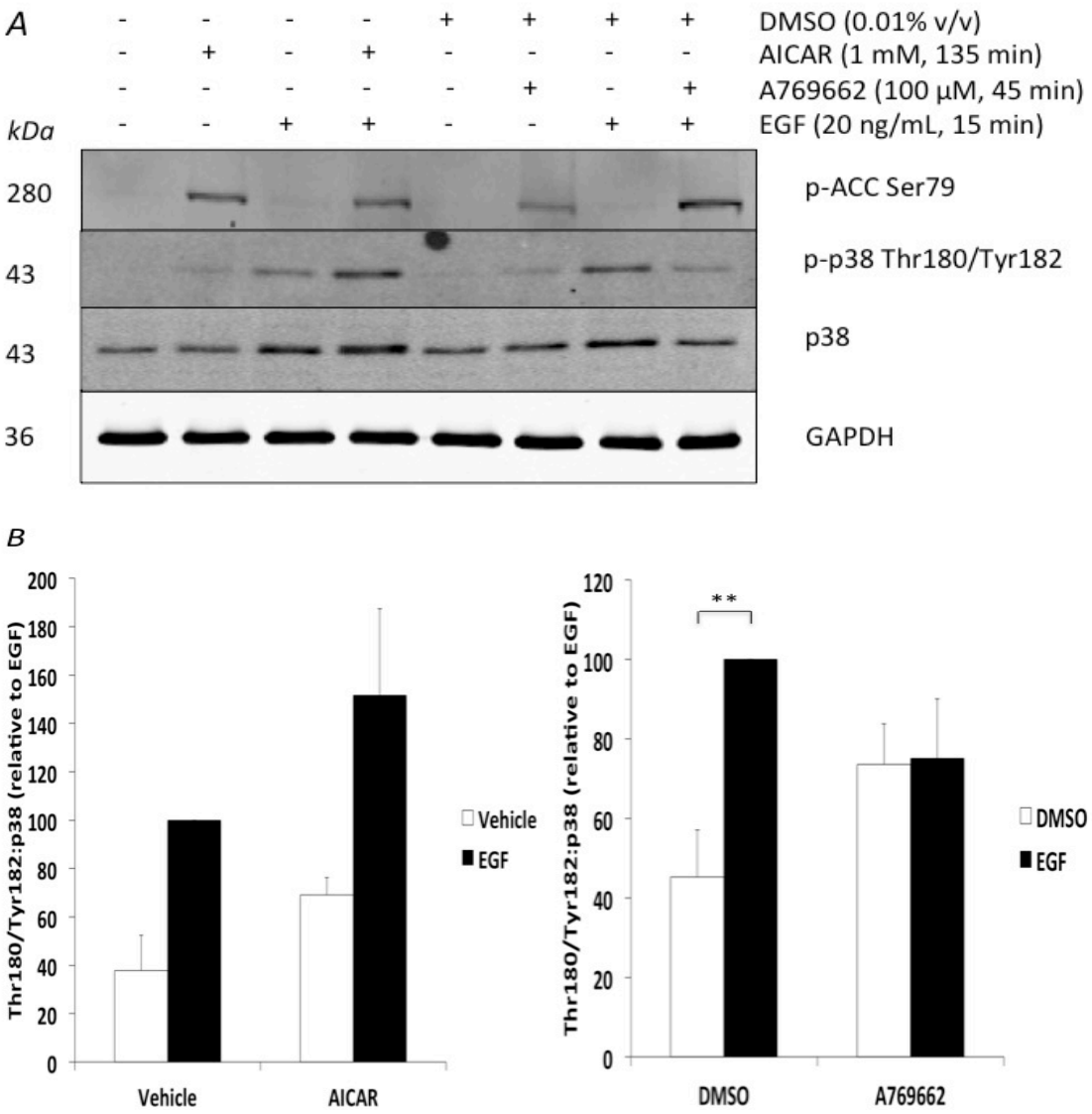


Figure 5.8 Effect of short-term incubation with AMPK activators on p38 phosphorylation in DU145 cells

DU145 cells were subjected to incubation for 2 h in serum-free medium before incubation with 1 mM AICAR (135 min) or 100 μM A769662 (45 min). EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of p38 phosphorylation level (**: $p<0.01$, N=3 for AICAR, N=6 for A769662).

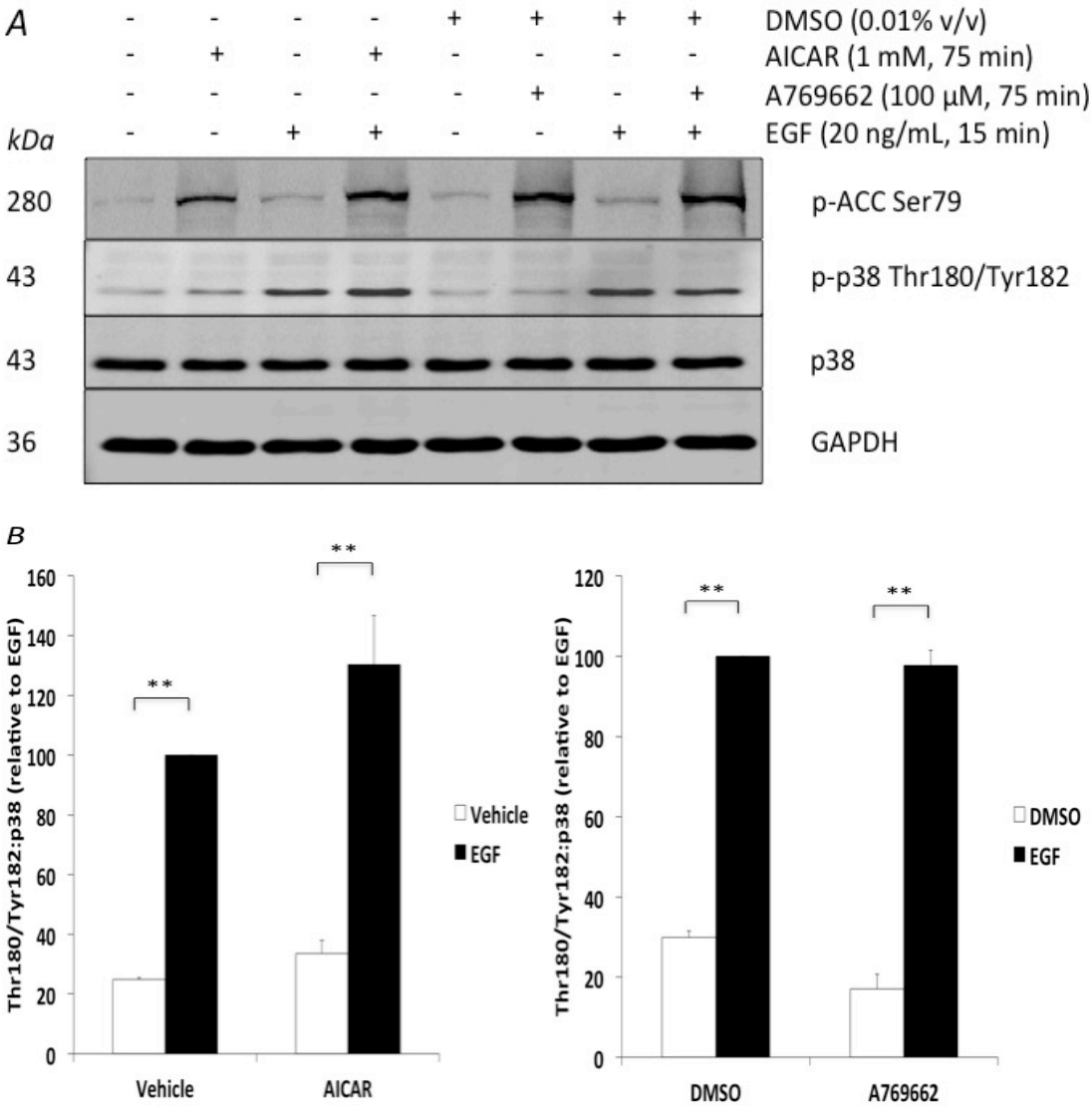


Figure 5.9 Effect of short-term incubation with AMPK activators on p38 phosphorylation in LNCaP cells

LNCaP cells were subjected to incubation for 2 h in serum-free medium before incubation with 1 mM AICAR (75 min) or 100 μM A769662 (75 min). EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of p38 phosphorylation level (**: $p<0.01$, N=3).

5.2.1.4 Effect of AMPK activators on ERK5 and MEK5 phosphorylation in prostate cancer cell lines

In all three PC cell lines, EGF stimulated phosphorylation of ERK5 (Figures 5.10-5.12). In PC3 (Figure 5.10) and DU145 cells (Figure 5.11), incubation with AICAR significantly decreased EGF-stimulated ERK5 phosphorylation, whereas AICAR had no effect on EGF-stimulated ERK5 phosphorylation in LNCaP cells (Figure 5.12). In PC3 cells, incubation with A769662 had no effect on EGF-stimulated ERK5 phosphorylation (Figure 5.10), yet A769662 significantly reduced EGF-stimulated ERK5 phosphorylation in both DU145 and LNCaP cells (Figures 5.11 and 5.12). Moreover, it was observed that AICAR could cause a band-shift in immunoreactive ERK5 in PC3 and DU145 cells (Figures 5.10 and 5.11). This AICAR-stimulated band-shift was not inhibited by compound C under conditions where AMPK activity was inhibited in both PC3 and DU145 cells (Figure 5.13).

In addition, the phosphorylation of MEK5, the upstream kinase of ERK5 was also assessed in PC3 and DU145 cells, but there was no obvious effect on phospho-MEK5 by EGF, AICAR or A769662 (Figures 5.10 and 5.11). The anti-MEK5 antibody also recognised multiple species in both PC3 and DU145 cells (Figures 5.10 and 5.11).

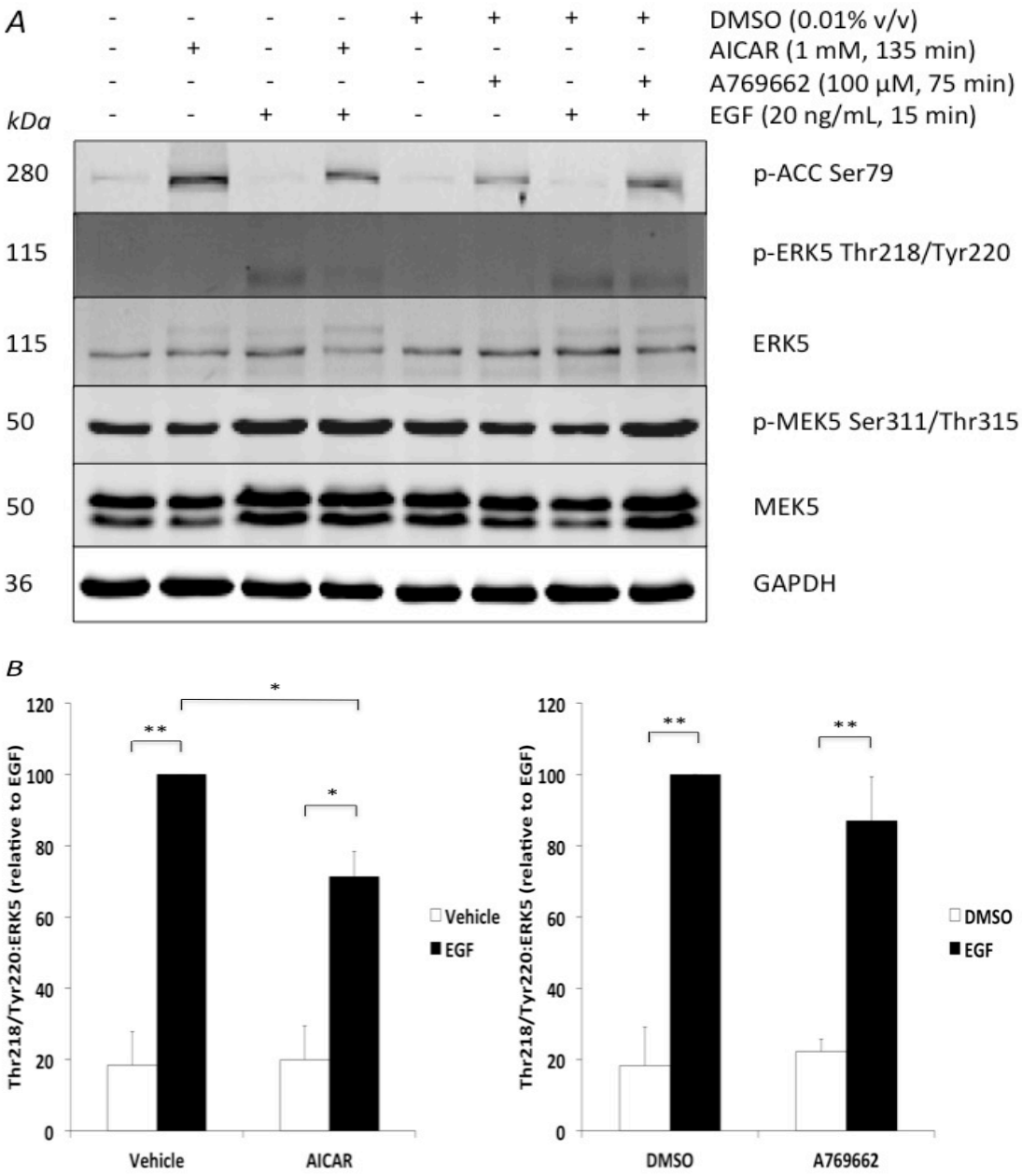


Figure 5.10 Effect of short-term incubation with AMPK activators on ERK5 and MEK5 phosphorylation in PC3 cells

PC3 cells were incubated for 2 h in serum-free medium before incubation with 1 mM AICAR (135 min) or 100 μM A769662 (75 min). EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of ERK5 phosphorylation level (*: $p<0.05$, N=3).

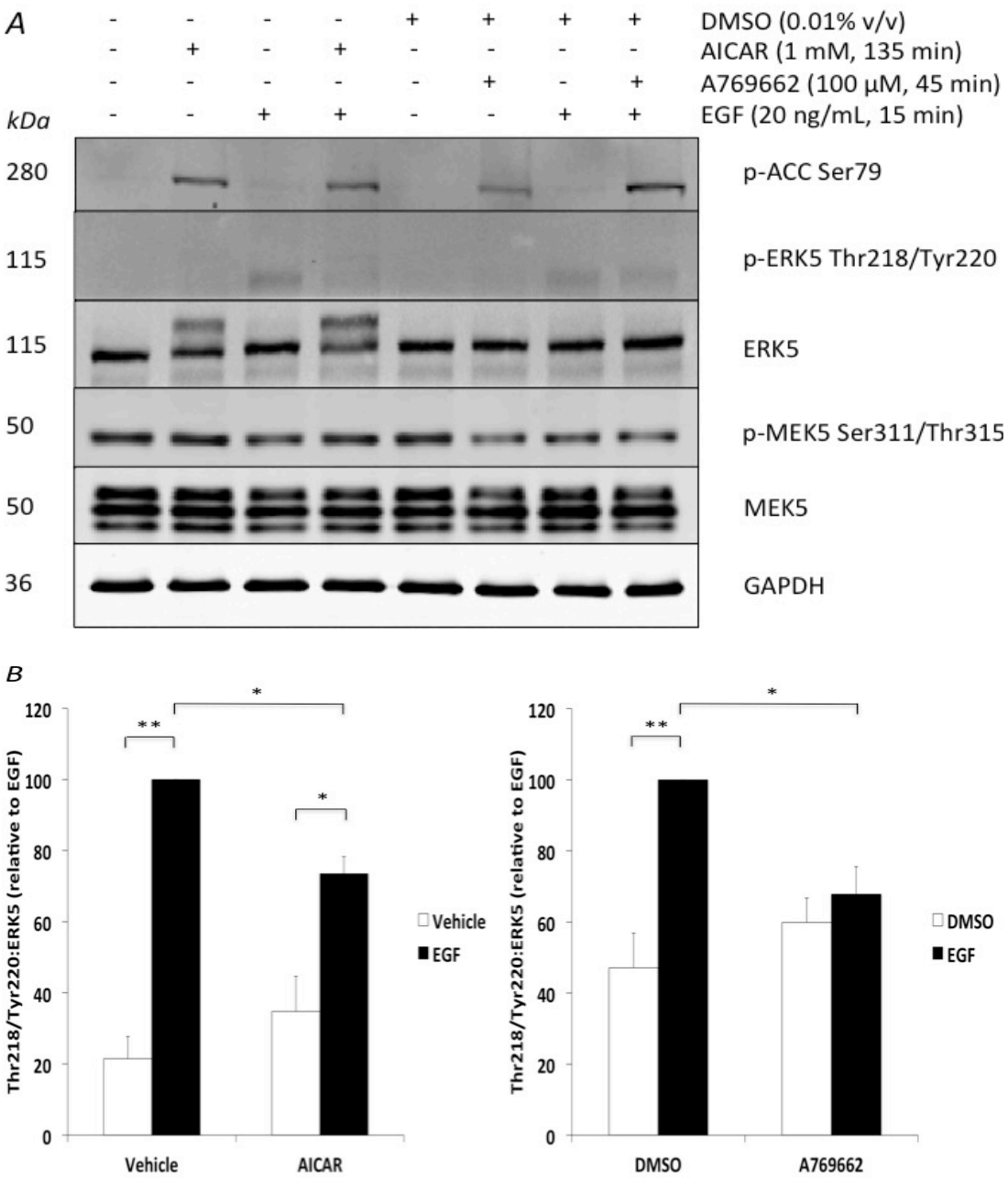


Figure 5.11 Effect of short-term incubation with AMPK activators on ERK5 and MEK5 phosphorylation in DU145 cells

DU145 cells were subjected to incubation for 2 h in serum-free medium before incubation with 1 mM AICAR (135 min) or 100 μM A769662 (45 min). EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of ERK5 phosphorylation level (*: $p<0.05$, **: $p<0.01$, N=3).

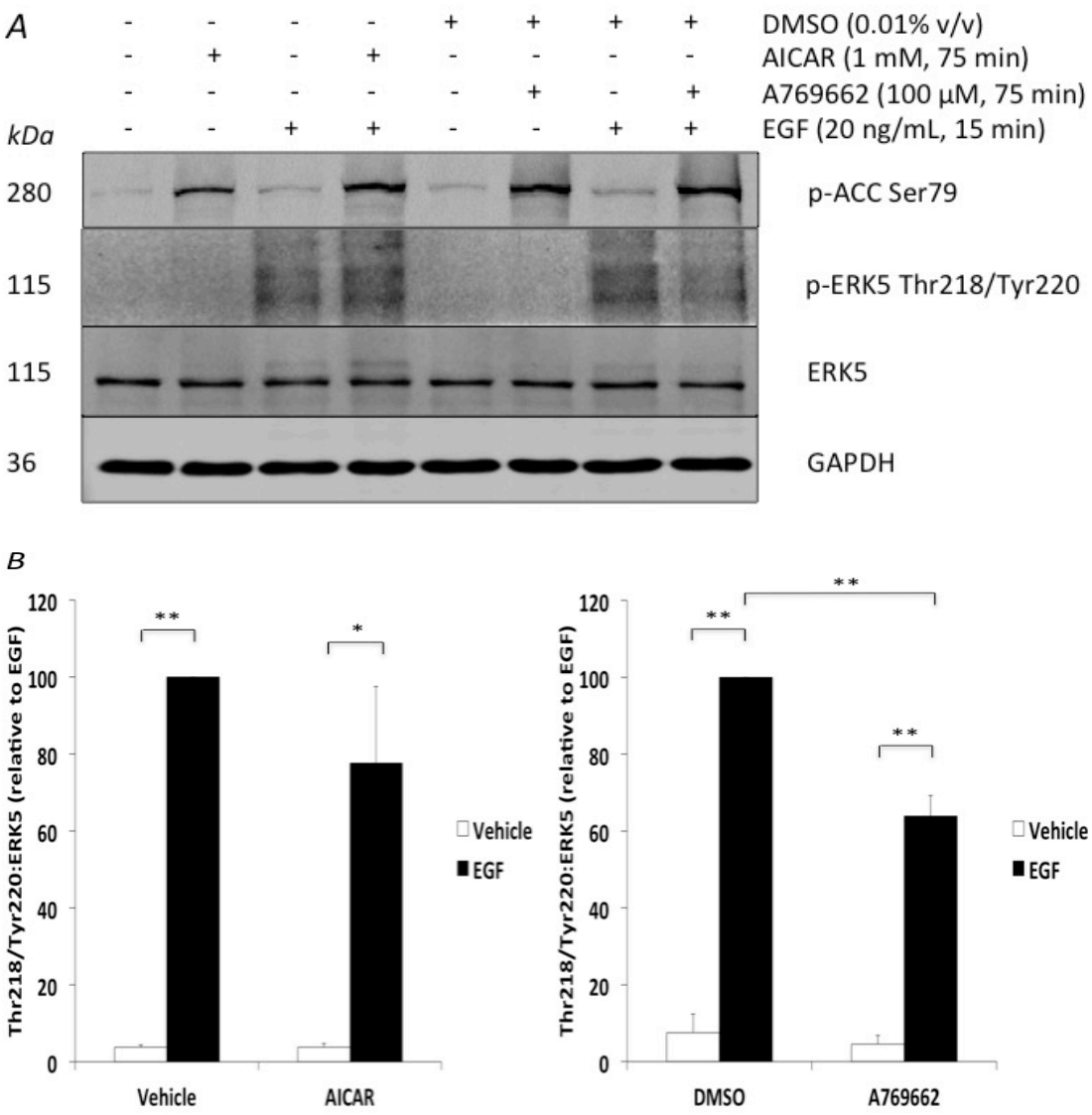


Figure 5.12 Effect of short-term incubation with AMPK activators on ERK5 phosphorylation in LNCaP cells

LNCaP cells were subjected to incubation for 2 h in serum-free medium before incubation with 1 mM AICAR (75 min) or 100 μM A769662 (75 min). EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of ERK5 phosphorylation level (*: $p<0.05$, **: $p<0.01$, N=3).

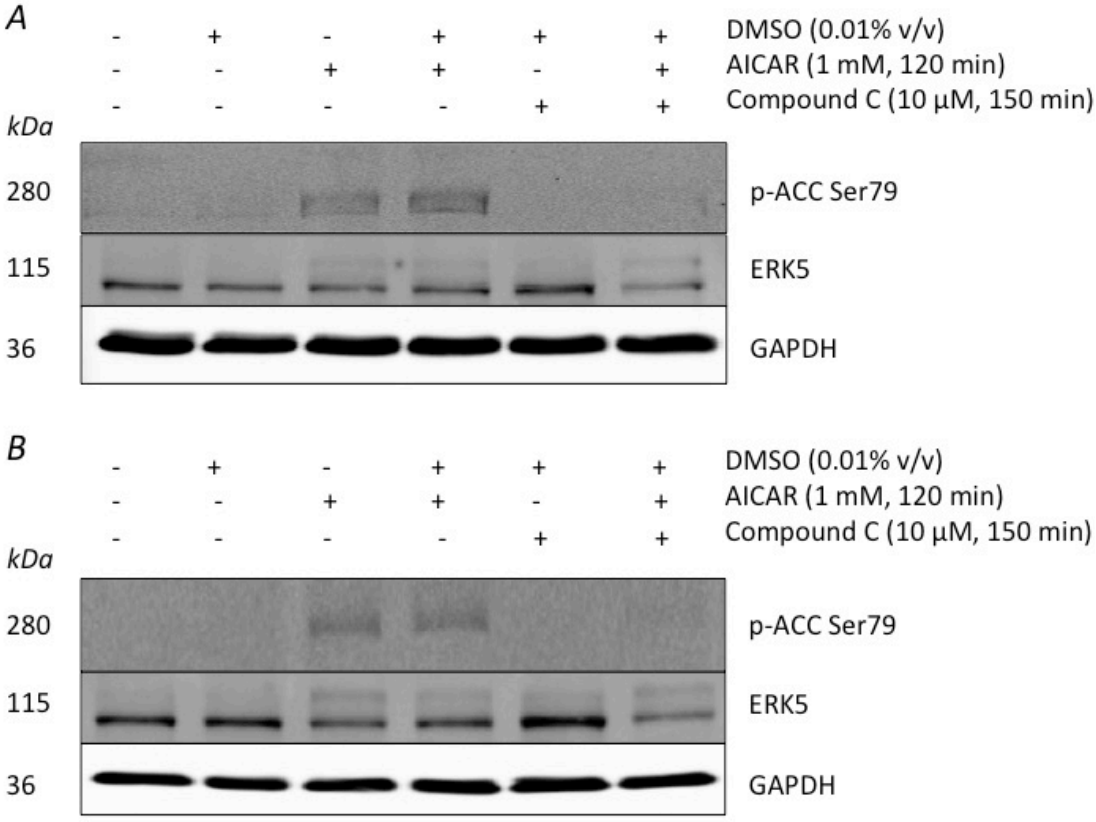


Figure 5.13 Effect of compound C on AICAR-mediated ERK5 band-shift in PC3 and DU145 cells

(A) PC3 cells or (B) DU145 cells were incubated for 2 h in serum-free medium before incubation with 10 μM compound C 30 min prior to stimulation with 1 mM AICAR (120 min). Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control.

5.2.2 Effect of AMPK activators on ERK5 phosphorylation in PC3 cells overexpressing ERK5 and MEK5

5.2.2.1 Role of AMPK on EGF-stimulated ERK5 phosphorylation in PC3 cells overexpressing ERK5

As the levels of EGF-stimulated ERK5 phosphorylation were relatively low in PC cell lines, the effect of AICAR and A769662 on EGF-stimulated ERK5 phosphorylation was assessed in PC3 cells stably expressing ERK5 (PC3-ERK5-18R-Flag). Stimulation with AMPK activators had similar effects on EGF-stimulated phospho-ERK5 to PC3 cells (Figure 5.14, compare with Figure 5.10), yet the extent of ERK5 phosphorylation was still very weak even in this cell line (Figure 5.14).

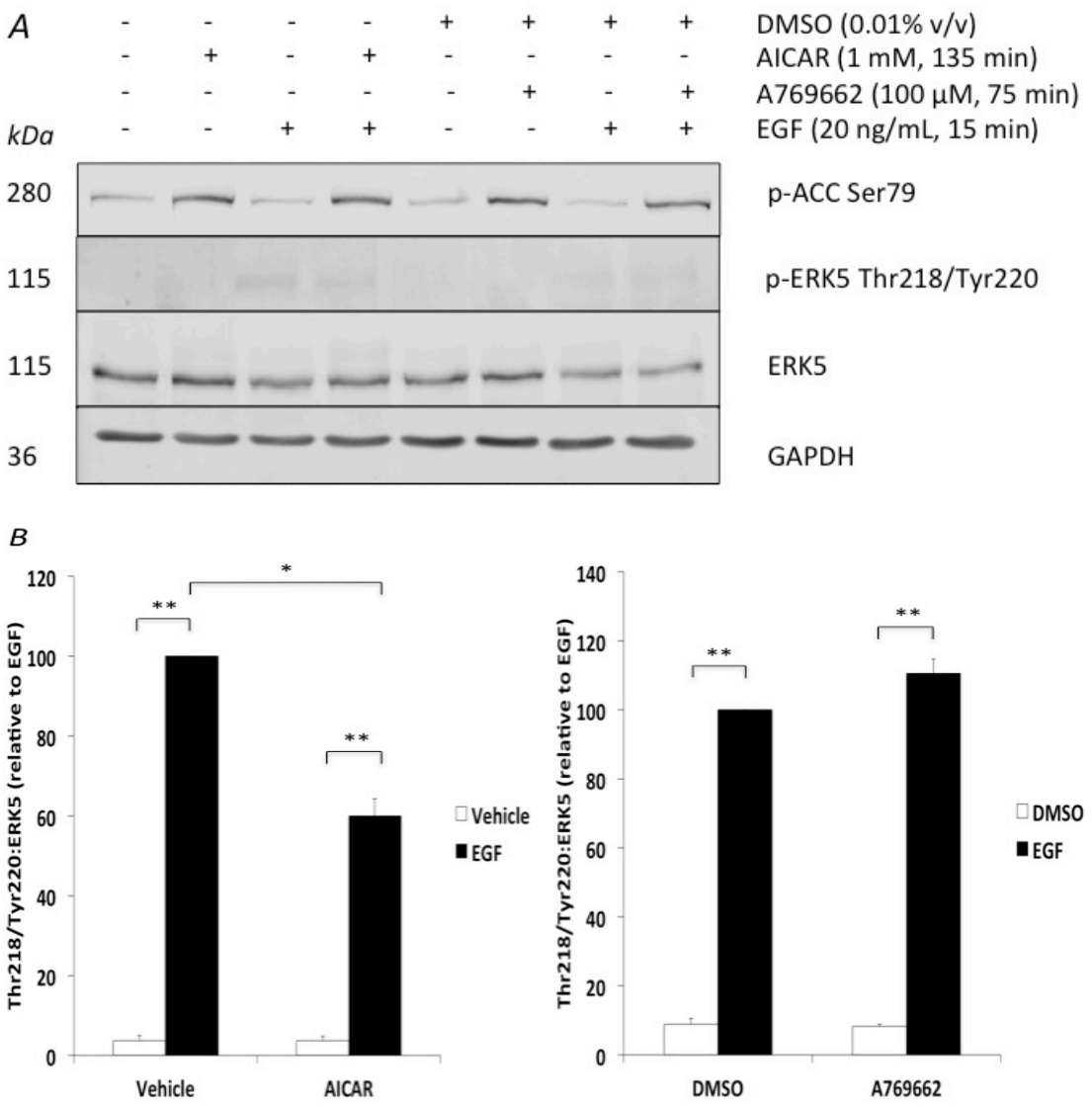


Figure 5.14 Effect of short-term incubation with AMPK activators on ERK5 phosphorylation in PC3 cells over-expressing ERK5

PC3-ERK5-18R-Flag cells were incubated for 2 h in serum-free medium before incubation with 1 mM AICAR (135 min) or 100 μM A769662 (75 min). EGF (20 ng/mL) was added 15 min prior to lysis. Protein Lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. Experiments were repeated for at least three times with the representative blots shown. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of ERK5 phosphorylation level (*: $p<0.05$, **: $p<0.01$, N=3).

5.2.2.2 Assessment of transient transfection with constitutively active MEK5 in PC3 cells overexpressing ERK5

To increase the extent of ERK5 phosphorylation, PC3 cells overexpressing ERK5 (clones PC3-ERK5-17P-Flag or PC3-ERK5-18R-Flag cells (McCracken *et al.*, 2008, Ramsay *et al.*, 2011)) or expressing empty vector were transiently transfected with plasmids expressing HA-tagged constitutively active MEK5, either MEK5D (MSCU-MEK5D) or MEK5DD (pCMV-MEK5DD-HA) as described in section 2.2.6. PC3-ERK5-18R-Flag cells, but not the related clone PC3-ERK5-17P-Flag cells had substantially increased ERK5 levels than PC3 cells expressing empty vector (Figure 5.15). Transient transfection with MEK5DD, but not MEK5D increased the levels of species of a lower molecular mass than endogenous MEK5 as assessed with both anti-MEK5 and anti-HA antibodies (Figure 5.15). Transfection of PC3-ERK5-18R-Flag cells with MEK5DD-HA stimulated a band shift in ERK5 and increased phospho-ERK5 immunoreactivity (Figure 5.15). These results indicate that use of PC3-ERK5-18R cells along with the MEK5DD plasmid provides the maximal detectable ERK5 phosphorylation with which to assess the effect of AMPK activators (Figure 5.15).

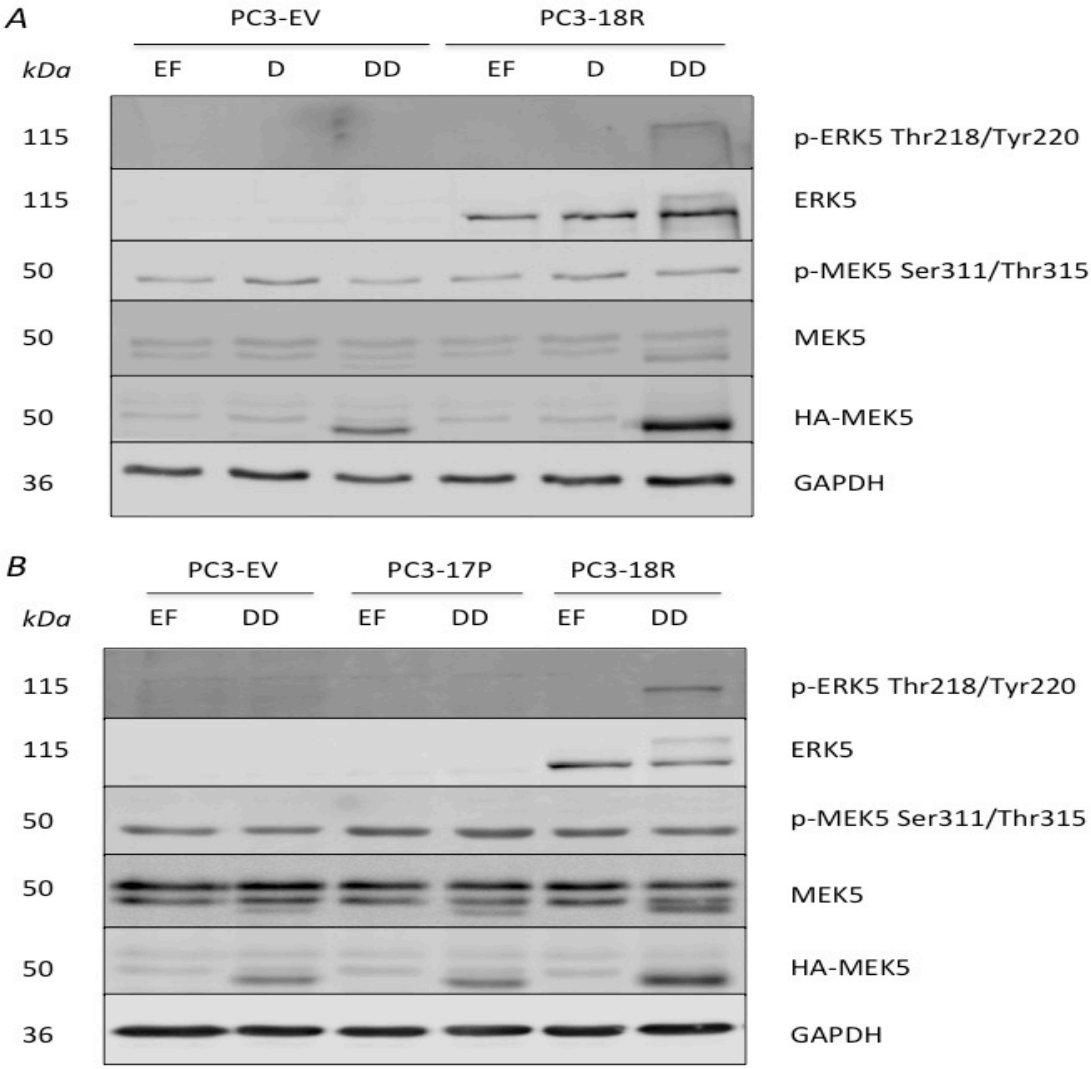


Figure 5.15 Transient transfection of PC3 cells with mutant active MEK5

PC3-EmptyVector (PC3-EV), PC3-ERK5-17P-Flag (PC3-17P) and PC3-ERK5-18R-Flag (PC3-18R) cells were harvested by trypsinisation and resuspended in Nucleofection Kit V solution. Plasmids (3 μg) pCMV2-EmptyVector-Flag (EF), MSCU-MEK5D (D) and pCMV-MEK5DD-HA (DD) in 100 μL of Kit V solution were added to cells and subjected to transfection program (T-013) using a Nucleofector II machine. Transfected cells were cultured for 72 h and lysates prepared. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Comparison of efficiency of plasmids in PC3-18R cells, experiments were repeated at least three times with representative blots shown. (B) Comparison of transient DD plasmid transfection using different PC3-ERK5 cells, experiments were repeated at least two times with representative blots shown.

5.2.2.3 Effect of AMPK activation on MEK5DD-induced ERK5 phosphorylation PC3-ERK5 cells

The effects of AMPK activators on either EGF or MEK5DD-induced ERK5 phosphorylation were analysed in PC3-ERK5-18R cells. Transient transfection of cells with MEK5DD caused a band shift with a higher molecular mass form of ERK5 appearing, that was recognised by the anti-phospho ERK5 antibody (Figures 5.16 and 5.17). Intriguingly, EGF-stimulated phospho-ERK5 immunoreactivity was different to MEK5DD-induced phospho-ERK5 immunoreactivity in three ways. Firstly, the molecular mass of EGF-stimulated phospho-ERK5 was lower than the mass of ERK5, whereas MEK5DD-induced phospho-ERK5 was of a higher molecular mass than ERK5. Secondly, the shape of the phospho-ERK5 species is broad and diffuse in EGF-stimulated cells compared to a thinner, more defined species in the MEK5DD-induced cells. Finally, preincubation with AICAR decreased EGF-stimulated phospho-ERK5 immunoreactivity, whereas neither AICAR nor A769662 had any effect on MEK5DD-induced phospho-ERK5 immunoreactivity (Figures 5.16 and 5.17).

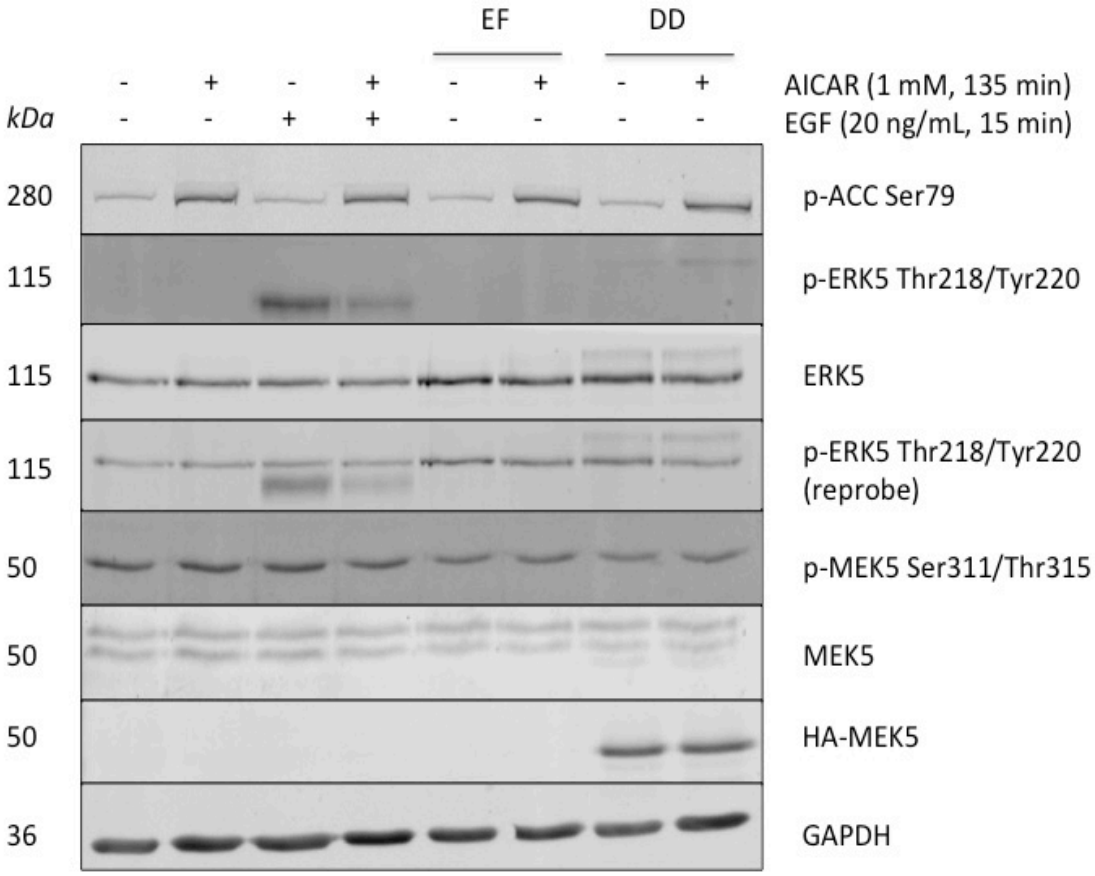


Figure 5.16 Effects of AICAR on transient MEK5 transfection in PC3-ERK5-18R cells

PC3-ERK5-18R-Flag cells were harvested by trypsinisation and resuspended in Nucleofection Kit V solution. Plasmids (3 μg) pCMV2-EmptyVector-Flag (EF) and pCMV-MEK5DD-HA (DD) in 100 μL of Kit V were added to cells and subjected to transfection program (T-013) using a Nucleofector II machine. Transfected cells were cultured for 72 h, incubated for 2 h in serum-free medium and stimulated with 1 mM AICAR for 135 min. EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. Experiments were repeated for at least three times with the representative blots shown.

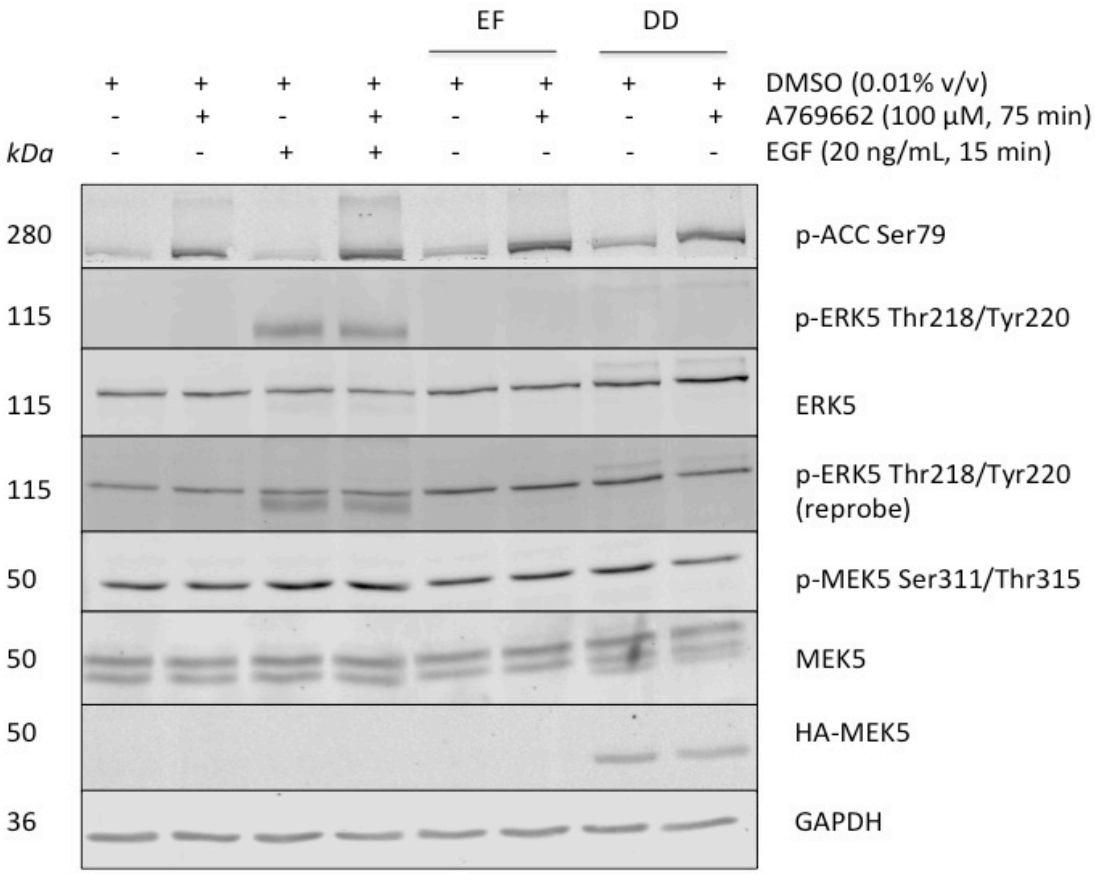


Figure 5.17 Effects of A769662 on transient MEK5 transfection in PC3-ERK5-18R cells

PC3-ERK5-18R-Flag cells were harvested by trypsinisation and resuspended in Nucleofection Kit V solution. Plasmids (3 µg) pCMV2-EmptyVector-Flag (EF) and pCMV-MEK5DD-HA (DD) in 100 µL of Kit V were added to cells and subjected to transfection program (T-013) using a Nucleofector II machine. Transfected cells were cultured for 72 h, incubated for 2 h in serum-free medium and stimulated with 100 µM A769662 for 75 min. EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. Experiments were repeated for at least three times with the representative blots shown.

5.2.3 Analysis of AMPK activation on EGF-stimulated MAPK signalling using wild type and AMPK $\alpha1^{-/-}$ AMPK $\alpha2^{-/-}$ mouse embryonic fibroblasts mouse embryonic fibroblasts

5.2.3.1 EGF-stimulated MAPK phosphorylation in mouse embryonic fibroblasts

To further examine the role of AMPK in MAPK signalling, WT and *AMPK $\alpha1^{-/-}$ AMPK $\alpha2^{-/-}$* KO mouse embryonic fibroblast (MEFs) were utilised. EGF rapidly stimulated phosphorylation of ERK1/2 and ERK5 in both WT and *AMPK $\alpha1^{-/-}$ AMPK $\alpha2^{-/-}$* KO MEFs (Figures 5.18). However, phosphorylation level of p38 and JNK was not obviously increased (data not shown).

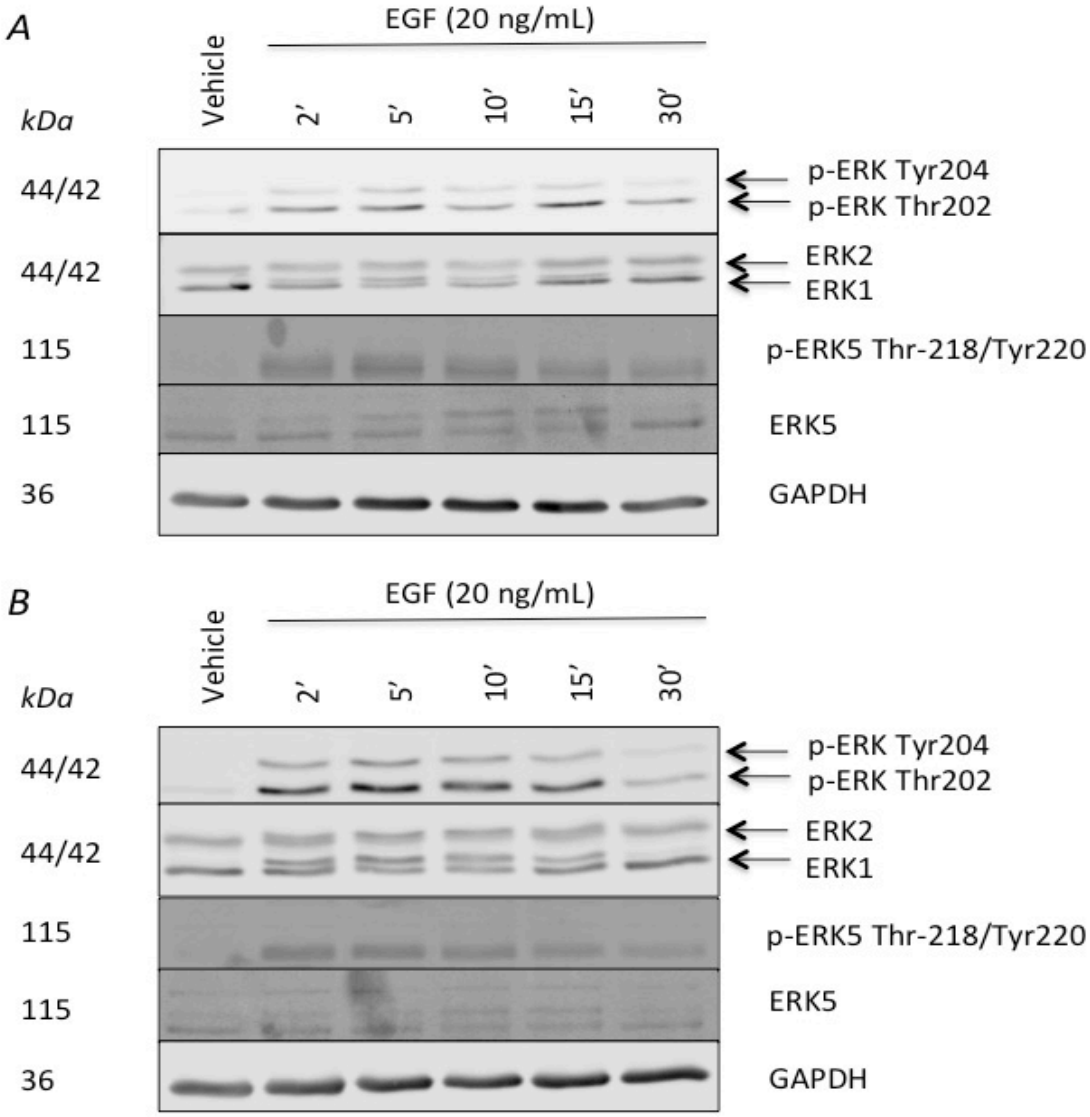


Figure 5.18 Stimulation of mitogen-activated protein kinase phosphorylation using EGF in wild type (WT) and *AMPK a1*$^{-/-}$ *AMPK a2*$^{-/-}$ knock out (KO) mouse embryonic fibroblasts (MEFs)

WT (A) and AMPK$^{-/-}$ KO (B) MEFs were incubated for 2 h in serum-free medium before stimulation with 20 ng/mL EGF for the indicated times and lysates prepared. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. Blots are representative of a single experiment.

5.2.3.2 Effect of AMPK activators on MAPK signalling in mouse embryonic fibroblasts

The effects of AICAR and A769662 on EGF-stimulated MAPK phosphorylation were assessed in both WT and *AMPK $\alpha1^{-/-}$ AMPK $\alpha2^{-/-}$* KO MEFs. It seems that A769662 prevented, at least partially, EGF-stimulated ERK5 phosphorylation in the WT MEFs, but not in the *AMPK $\alpha1^{-/-}$ AMPK $\alpha2^{-/-}$* KO MEFs. Neither AICAR nor A769962 had any effect on basal or EGF-stimulated ERK1/2 in either WT or *AMPK $\alpha1^{-/-}$ AMPK $\alpha2^{-/-}$* KO MEFs. (Figures 5.19 and 5.20) For JNK and p38, it is difficult to draw any conclusion regard the effect of AICAR or A769662 due to the low phosphorylation level even after EGF stimulation (data not shown).

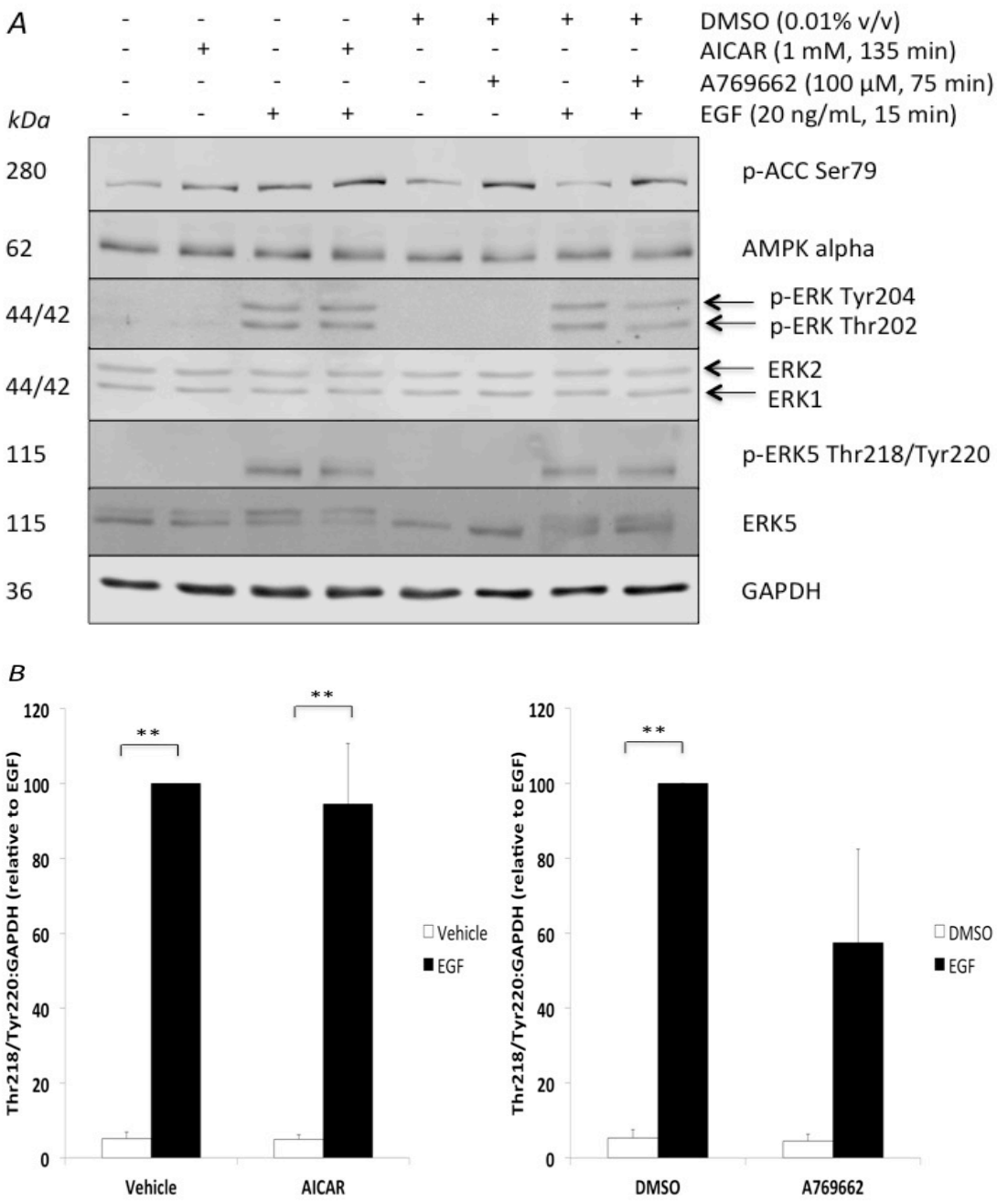


Figure 5.19 Effect of AMPK activators on EGF-stimulated mitogen-activated protein kinase phosphorylation in wild type (WT) mouse embryonic fibroblasts (MEFs)

WT MEFs were incubated for 2 h in serum-free medium before stimulation with 1 mM AICAR (135 min) or 100 µM A769662 (75 min). EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots (N=3). (B) Densitometric analysis of ERK5 phosphorylation level (**: $p<0.01$, N=3).

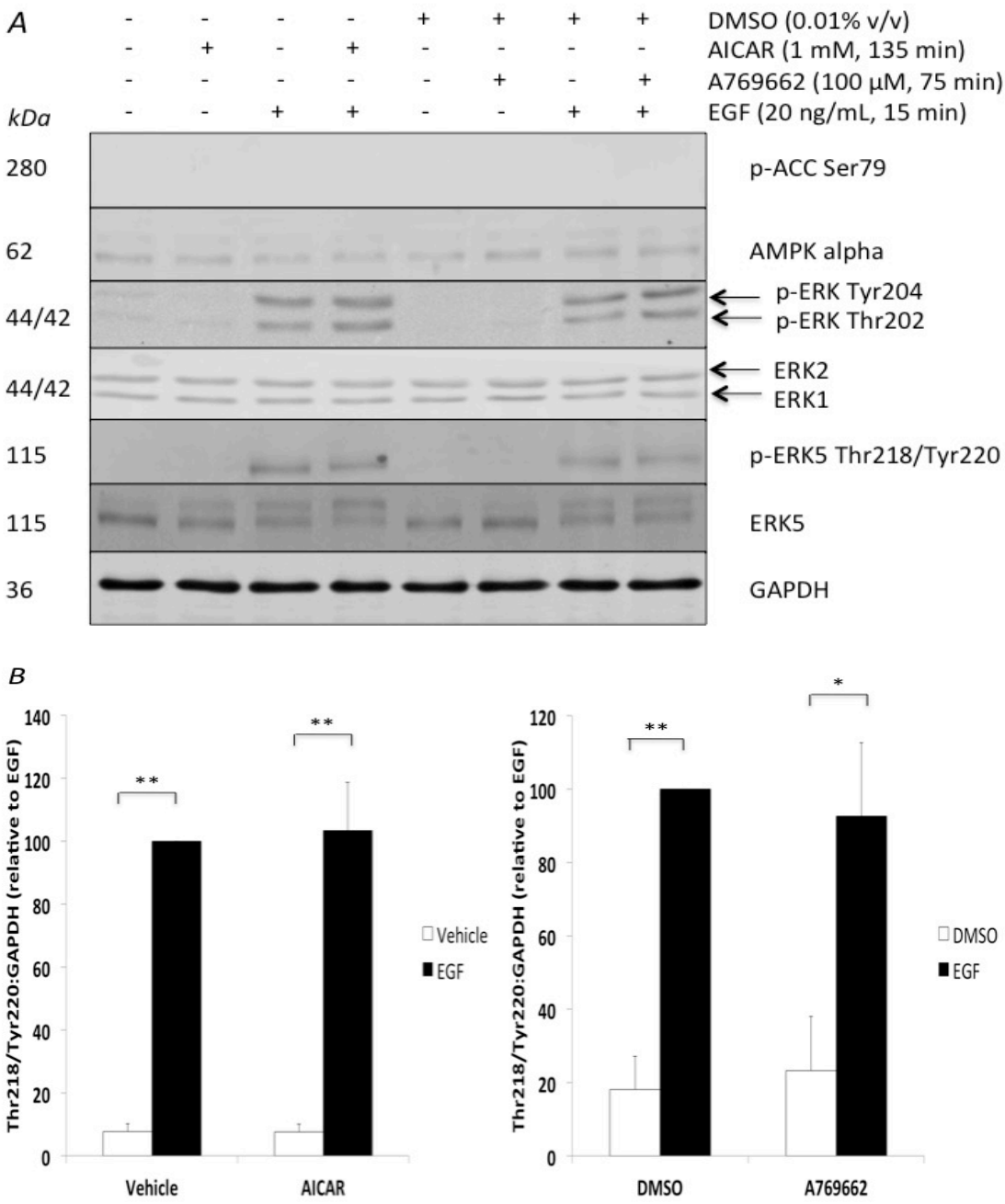


Figure 5.20 Effect of AMPK activators on EGF-stimulated mitogen-activated protein kinase phosphorylation in *AMPK α1*$^{-/-}$ *AMPK α2*$^{-/-}$ knock out (KO) mouse embryonic fibroblasts (MEFs)

AMPK$^{-/-}$ KO MEFs were incubated for 2 h in serum-free medium before stimulation with 1mM AICAR (135 min) or 100 μM A769662 (75 min). EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of ERK5 phosphorylation level (*: $p<0.05$, **: $p<0.01$, N=3).

5.3 Discussion

ERK1/2 plays an important role in terms of proliferation, differentiation and migration in carcinogenesis (Dhillon *et al.*, 2007). The ERK1/2 pathway is usually altered in cancer (Dhillon *et al.*, 2007), including activation in prostate animal model and clinical samples (Uzgare *et al.*, 2003). Currently, little is known about the role of JNK pathway in cancer (Dhillon *et al.*, 2007). It was reported that JNK was inactivated in animal models and clinical PC samples (Uzgare *et al.*, 2003). However, other studies indicate JNK can be phosphorylated upon EGF-stimulation in SiHa human cervical cancer cells (Liu *et al.*, 2001). In this study, EGF was unable to stimulate JNK in all of the PC cell lines used. The data in this chapter demonstrate that long-term incubation with A769662 decreased ERK1 phosphorylation in PC3 cells. Short-term incubation with AMPK activators had no consistent effect, however, on EGF-stimulated ERK1/2, JNK or p38 phosphorylation in PC3, DU145 or LNCaP cells. As long- but not short-term activation of AMPK inhibited ERK1/2 signalling, this may reflect that AMPK activators inhibit ERK1/2 signalling indirectly, rather than rapid phosphorylation of a component of the MAPKKK-MEK1/2-ERK1/2 pathway. Furthermore, A769662 stimulated ERK1/2 phosphorylation in DU145 cells, although this seems unlikely to be AMPK-dependent as compound C cannot eliminate this effect. This result indicates that A769662 might have an off-target effect on ERK1/2 signalling in these cells in addition to its role as an AMPK activator. It was shown in one study recently that ERK1/2 could inhibit AMPK activity by phosphorylate Ser485 *in vitro* (Lopez-Cotarelo *et al.*, 2015). Therefore further study can be carried out to determine the signalling loop between ERK1/2 and AMPK.

The p38 pathway is important in cancer apoptosis, cell cycle control, growth and differentiation (Dhillon *et al.*, 2007). It is also associated with proliferation in PC (Uzgare *et al.*, 2003). The current study demonstrates that EGF has variable effects on p38 phosphorylation when comparing PC3, DU145 and LNCaP cells, yet neither AICAR nor A769662 had any consistent effect on p38 phosphorylation in these cell lines. AMPK activators have been demonstrated to inhibit inflammatory

MAPK activation (Su *et al.*, 2007, Jeong *et al.*, 2009, Green *et al.*, 2011b), such that it is possible that AMPK specifically suppresses MAPK activation in response to inflammatory rather than growth factor stimuli. It is also possible that the effect produced here by AMPK activators on EGF-stimulated MAPK phosphorylation is a stimulus specific observation.

ERK5 has been proved as an important kinase involved in PC, especially in the invasive phenotype (McCracken *et al.*, 2008, Ramsay *et al.*, 2011). The MEK5/ERK5 signalling cascade is a potential therapeutic target in PC, yet the mechanism(s) regulating ERK5 are not fully understood. Previously, it has been suggested that AMPK might be an upstream kinase of ERK5 (Young *et al.*, 2009). The results presented here suggested that AICAR decreased EGF-stimulated ERK5 phosphorylation in PC3 and DU145 but not in LNCaP cells. A769662 on the other hand decreased EGF-stimulated ERK5 phosphorylation in DU145 and LNCaP cells, but not in PC3 cells. These data suggest that AMPK activation may have an effect on ERK5 phosphorylation that might be cell-specific. Taken together, these data suggest that AMPK influence EGF-stimulated ERK5 phosphorylation. Firstly, the AMPK activators used in the above experiments are structurally unrelated and act through different mechanisms, and yet a generally inhibition of EGF-stimulated ERK5 phosphorylation is observed in PC cell lines. Secondly, the fact that A769662 inhibited EGF-stimulated ERK5 phosphorylation in the WT MEFs but not the *AMPK* $\alpha 1^{-/-}$ *AMPK* $\alpha 2^{-/-}$ KO MEFs is another strong evidence towards an AMPK dependent mechanism. Another interesting finding is a band shift in detected by the anti-ERK5 antibody after incubation with AICAR alone in both PC3 and DU145 cells. This effect is likely to be independent of AMPK activation, since the effect can still be reproduced after preincubation with compound C. The upstream kinase of ERK5, MEK5 was also analysed in the current study. There are two MEK5 isoforms - the α isoform (50 kDa) and β isoform (40 kDa) (English *et al.*, 1995). It is reported that MEK5α expression is higher in cancer cell lines including DU145, and that MEK5α is the isoform responsible for ERK5 activation and nuclear translocation (Cameron *et al.*, 2004). Therefore, the α isoform is more important in terms of MEK5/ERK5 signalling. In the current study, three species were obtained when immunoblots were probed

with the anti-MEK5 antibody in DU145 cells, but only two species were observed in PC3 cells with the same antibody. All of the species observed were all well above 50kDa, suggesting that they are unlikely to represent different MEK5 isoforms. Similarly, it is unlikely that the multiple species can be explained by ERK5 splicing since there is only one form of ERK5 in humans (Lee *et al.*, 1995), despite alternative splicing occurring in the mouse (Yan *et al.*, 2001). In order to explore the AICAR-mediated band shift in ERK5 and inhibition of ERK5 phosphorylation by AMPK activators, PC3-18R-ERK5-Flag cells were used. This cell line overexpresses ERK5 and has been utilised as a tool in previous studies (Ramsay *et al.*, 2011). The data presented here demonstrates that the use of pCMV-MEK5DD-HA plasmid in PC3-18R-ERK5-Flag cells led to robust ERK5 phosphorylation confirmed by both the anti-phospho-ERK5 antibody and the band shift detected by the anti-ERK5 antibody. Interestingly, however, the EGF-stimulated phospho-ERK5 immunoreactivity is distinct from that elicited by pCMV-MEK5DD-HA in terms of species size and shape. EGF-stimulated phospho-ERK5 has a smaller mass (below 115 kDa) and is diffuse in shape. The MEK5DD-stimulated phospho-ERK5, in contrast, is of greater molecular mass (above 115 kDa) and more focused in shape. In addition, AICAR had no effect on the ERK5 phosphorylation level induced by MEK5DD. These data might suggest that EGF-stimulated ERK5 phosphorylation through a non-cardinal pathway independent from MEK5, and this mechanism is inhibited upon AICAR incubation. And this AICAR-induced inhibitory effect is likely to be AMPK-dependent giving the above-mentioned evidence. Nonetheless, similar experiments using A769662 showed no effect on MEK5DD induced ERK5 phosphorylation level.

Indeed, most of the data presented in this chapter suggest that AMPK activation is associated with reduced EGF-stimulated ERK5 signalling. But AMPK activation cannot reduce phosphorylation of ERK5 by constitutively active MEK5. This indicates that AMPK does reduce ERK5 phosphorylation in response to EGF, possibly by stimulating a phosphatase that dephosphorylates ERK5, or it could act at MEK5 or upstream of MEK5. Since a constitutive MEK5 is used, AMPK may not be able to inhibit at this level. The findings on ERK5 in this project are novel as it showed for the first time that AMPK activators could decrease ERK5

phosphorylation in PC cells possibly in an AMPK-related mechanism. It also suggests that these effects might be independent from the classical MEK5/ERK5 signalling cascade. In order to understand the full mechanism, further investigation is required to underpin the immunoreactivity of EGF-stimulated phospho-ERK5 first.

Chapter 6. Effect of AMPK activators on EGF-stimulated Akt signalling in human prostate cancer cell lines

6.1 Introduction

6.1.1 Brief overview of the PI3K/Akt signalling pathway

The PI3K/Akt signalling cascade is responsible for many crucial cellular functions such as growth, proliferation, differentiation, survival and motility. After activation by growth factor binding to RTKs at the cell membrane, PI3K catalyses PIP3 synthesis from phosphatidylinositol-4,5-bisphosphate. PIP3 then recruits the serine/threonine protein kinase Akt to the plasma membrane, which is subsequently activated through phosphorylation by phosphoinositide-dependent kinase 1 and mTORC2. Active Akt can subsequently activate mTOR (Manning *et al.*, 2002, Luo *et al.*, 2003, Hemmings and Restuccia, 2012). PTEN, a tumour suppressor, which is found to be mutated in several cancer types (Li and Sun, 1997, Li *et al.*, 1997, Steck *et al.*, 1997), has been recognised as a negative regulator of the PI3K/Akt pathway by dephosphorylating PIP3, thereby terminating signalling (Sun *et al.*, 1999, Mills *et al.*, 2001, DeGraffenried *et al.*, 2004).

6.1.2 The role of the PI3K/Akt pathway in cancer

The PI3K/Akt signalling pathway is considered to be an important pathway that is hyperactivated in carcinogenesis (Staal, 1987, Brazil and Hemmings, 2001, Yap *et al.*, 2008, Hemmings and Restuccia, 2012). The PI3K/Akt pathway is also more frequently activated than any other pathway in tumours of cancer patients (Hennessy *et al.*, 2005) and has been researched thoroughly as a potential therapeutic target for human cancer (Vivanco and Sawyers, 2002, Luo *et al.*, 2003, Hennessy *et al.*, 2005, Yap *et al.*, 2008). It has been reported that genetic abnormalities of the PI3K/Akt pathway could contribute to tumourigenesis in many human cancers including glioblastoma, gliosarcoma and leukaemia as well as prostate, breast, colorectal, gastric, lung, hepatocellular, thyroid, endometrial, oesophageal, nasopharyngeal, cervical, uterine, ovarian and pancreatic cancers (van Dam *et al.*, 1994, Bellacosa *et al.*, 1995, Cheng *et al.*, 1996, Dahia *et al.*, 1997, Li *et al.*, 1997, Halachmi *et al.*, 1998, Kohno *et al.*, 1998, Nakatani *et al.*, 1999,

Harima *et al.*, 2001, Philp *et al.*, 2001, Actor *et al.*, 2002, Byun *et al.*, 2003, Knobbe and Reifenberger, 2003, Campbell *et al.*, 2004, Mizoguchi *et al.*, 2004, Samuels *et al.*, 2004, Oda *et al.*, 2005, Wu *et al.*, 2005, Bertelsen *et al.*, 2006, Douglas *et al.*, 2006, Gallia *et al.*, 2006, Livasy *et al.*, 2006, Nakayama *et al.*, 2006, Phillips *et al.*, 2006, Carpten *et al.*, 2007, Hollestelle *et al.*, 2007, Ollikainen *et al.*, 2007, Willner *et al.*, 2007). The most common genes mutated in the PI3K/Akt pathway are those that encode subunits of PI3K (*PIK3CA*, *PIK3R1*), *PTEN*, Akt isoforms (*AKT1*, *AKT2*, *AKT3*), PDK1 and RTKs that activate PI3K (*ERBB2*, *EGFR*) (Yap *et al.*, 2008, Courtney *et al.*, 2010). Several pharmacological agents developed to target the PI3K/Akt pathway have either undergone or are currently evaluated in clinical trials either alone or in combination, including PI3K inhibitors, Akt inhibitors, mTOR inhibitors and EGFR inhibitors (Yap *et al.*, 2008, Engelman, 2009, Courtney *et al.*, 2010). Furthermore, different models and therapeutic strategies being investigated trying to develop an efficient approach in targeting the PI3K/Akt signalling pathway (Wong *et al.*, 2010). Evidence has suggested that combination of PI3K/Akt inhibition with inhibition of other pathways may increase efficiency and with acceptable side effect profiles. For example the inhibition of both PI3K and MEK is more efficient than the inhibition of either pathway alone in cancer cell models (She *et al.*, 2005). Importantly, combining PI3K and MEK inhibitors has been reported to increase therapeutic efficiency, and yet reduce toxicity both *in vitro* and *in vivo* (Engelman *et al.*, 2008, Wong *et al.*, 2010), which would be explained by crosstalk between AMPK and the PI3K/Akt pathway. AMPK may interact with the PI3K pathway in a complex manner (Memmott and Dennis, 2009, Green *et al.*, 2011a). For example, it is established that AMPK inhibits mTOR (Sabatini, 2006). In one study, it is demonstrated that AMPK could stimulate PI3K/Akt in adipocytes *in vitro* (Tao *et al.*, 2010). The mechanism of mTOR inhibition is phosphorylation of TSC2 and raptor, which leads to mTORC1 inhibition (Inoki *et al.*, 2003, Gwinn *et al.*, 2008). On the other hand, Akt may also regulate AMPK activity by altering cellular ATP level, as MEFs lacking Akt exhibited increased AMP:ATP and AMPK activation (Hahn-Windgassen *et al.*, 2005). Furthermore, there are ample evidence that suggest phosphorylation of AMPK α1 at the inhibitory Ser485 site by Akt, leading to reduced AMPK activity in

several different cell types *in vitro* (Kovacic *et al.*, 2003, Horman *et al.*, 2006, Berggreen *et al.*, 2009, Ning *et al.*, 2011, Valentine *et al.*, 2014).

6.1.3 Akt signalling in prostate cancer

Gene mutation or deletion leading to activation of the PI3K/Akt pathway is the most common genetic defect in PC (Epstein and Lotan, 2015). In prostate cancer (PC), it has been reported that inactivating mutations of the *PTEN* gene leads to over-activation of Akt (Li *et al.*, 1997, Nakatani *et al.*, 1999). The role of AR mutation has long been recognised as an important driver (progression factor) in PC, especially in CRPC (Scher and Sawyers, 2005). The PI3K/Akt pathway and the AR are linked in a reciprocal way such that when one is inhibited, the other is activated so that tumour viability can be maintained (Carver *et al.*, 2011). Therefore, inhibition of both pathways at the same time might be necessary in order to enhance therapeutic efficiency (Carver *et al.*, 2011). Using animal models, it has also been demonstrated that the LKB1-AMPK pathway is important in suppressing tumourigenesis in PTEN-deficient tumours (Huang *et al.*, 2008).

6.1.4 EGF as a stimulus of Akt

EGF has been recognised as one of the key activators of the PI3K/Akt pathway (Prigent and Gullick, 1994), mediated by EGFR, which belongs to a family of four different ErbB receptors (Normanno *et al.*, 2006). In PC cells, EGFR is highly expressed in both PC3 and DU145 cells, and EGF stimulates phosphorylation of Akt in these cell lines (Gan *et al.*, 2010). More importantly, it was also demonstrated that EGF-stimulated Akt phosphorylation has a critical role in PC migration *in vitro* (Gan *et al.*, 2010). As shown by the apoptosis signalling array in Chapter 4, AMPK activators suppressed EGF-stimulated phospho-Akt Ser473 in PC3 cells after long-term incubation (72 h) (Figure 4.17). The work described in this study extends these findings to further assess the effects of AMPK activators on Akt phosphorylation in PC cell lines.

6.2 Results

6.2.1 Effect of AMPK activation on EGF-stimulated Akt signalling in PC3 cells

In agreement with the apoptosis signalling array data presented in Chapter 4, EGF rapidly stimulated phosphorylation of Akt at both Thr308 and Ser473 in PC3 cells (Figure 6.1). In addition, after 72 h preincubation with either AICAR or A769662, both EGF-stimulated phospho-Akt Thr308 and Ser473 phosphorylation was decreased, although only the decrease in phospho-Akt Ser473 level achieved statistical significance. ACC phosphorylation in response to both AICAR and A769662 was maintained for 72 h (Figure 6.1).

To examine whether AMPK activators rapidly inhibited EGF-stimulated Akt phosphorylation, similar experiments were conducted in which cells were preincubated with AMPK activators for shorter durations (135 min). Interestingly, incubation with AICAR had no effect on EGF-stimulated phosphorylation of Akt at Thr308 or Ser473, despite robustly stimulating ACC phosphorylation. Preincubation with A769662 did, however, significantly reduce EGF-stimulated phosphorylation of Akt Thr308 and Ser473 (Figure 6.2). The basal Ser473 phosphorylation level in response to AICAR is also slightly increased in PC3 cells (Figure 6.2). Using two isogenic cell lines PC3 and PC3M in parallel, experiments were carried out to determine the effect of AMPK activators on Akt Ser473 phosphorylation. Phosphorylation of Ser473 can be seen after short-term incubation with AICAR in both PC3 and PC3M cells (Figure 6.3). A769662, on the other hand, decreased basal phospho-Akt Ser473 levels in PC3 cells but increased basal phospho-Akt Ser473 level in PC3M cells at a concentration of 50 μM (Figure 6.3). Down-regulation of AMPK using *PRKAA1* siRNA had no effect on phospho-Akt Ser473 level in PC3 cells, and inhibition of Akt using the PI3K inhibitor LY294002 did not have any additive impact with the anti-proliferative effects of AICAR in PC3M cells (Figure 6.3). Whether these effects produced were AMPK-dependent remain to be tested.

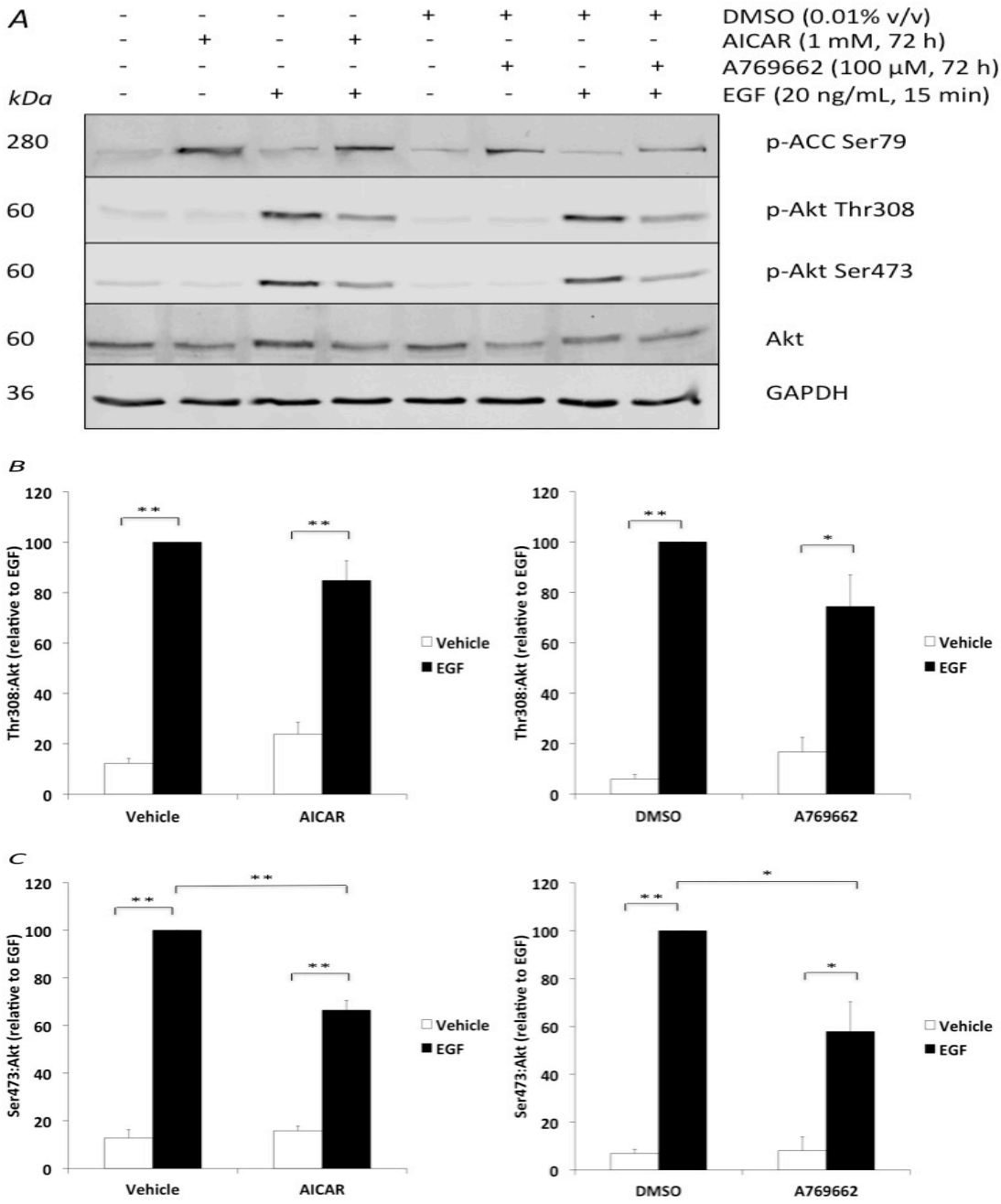


Figure 6.1 Effect of 72h incubation with AMPK activators on EGF-stimulated Akt phosphorylation in PC3 cells

PC3 cells were incubated for 2 h in serum-free medium before incubation with 1 mM AICAR or 100 μM A769662 for 72 h. EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of phospho-Akt Thr308 level (**: $p<0.01$, N=3). (C) Densitometric analysis of phospho-Akt Ser473 level (*: $p<0.05$, **: $p<0.01$, N=3).

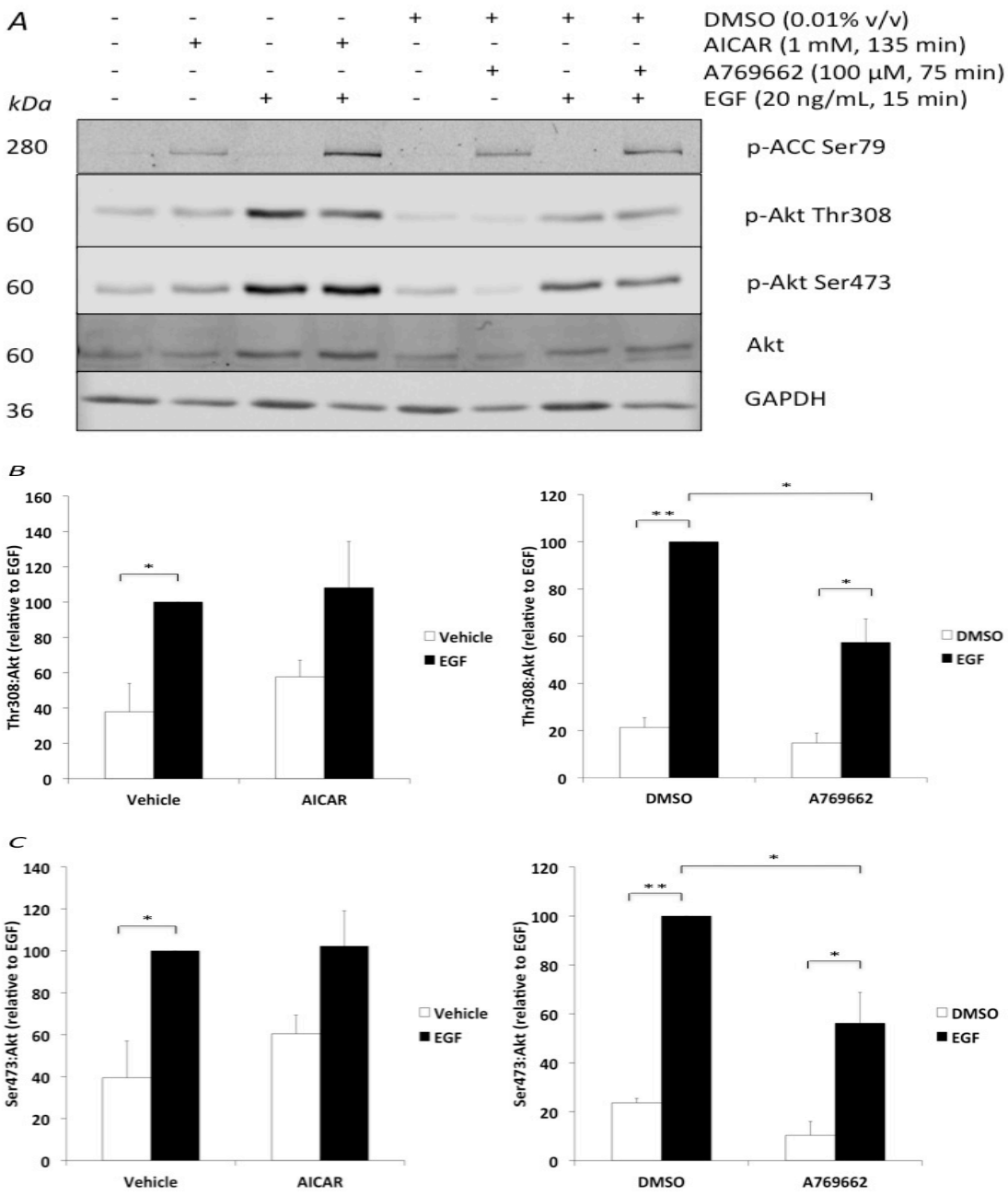


Figure 6.2 Effects of short-term incubation with AMPK activators on EGF-stimulated Akt phosphorylation in PC3 cells

PC3 cells were incubated for 2 h in serum-free medium before incubation with 1 mM AICAR (135 min) or 100 μM A769662 (75 min). EGF (20 ng/mL) was added 15 min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A) Representative blots. (B) Densitometric analysis of phospho-Akt Thr308 level (**: $p<0.01$, N=3). (C) Densitometric analysis of phospho-Akt Ser473 level (**: $p<0.01$, N=3).

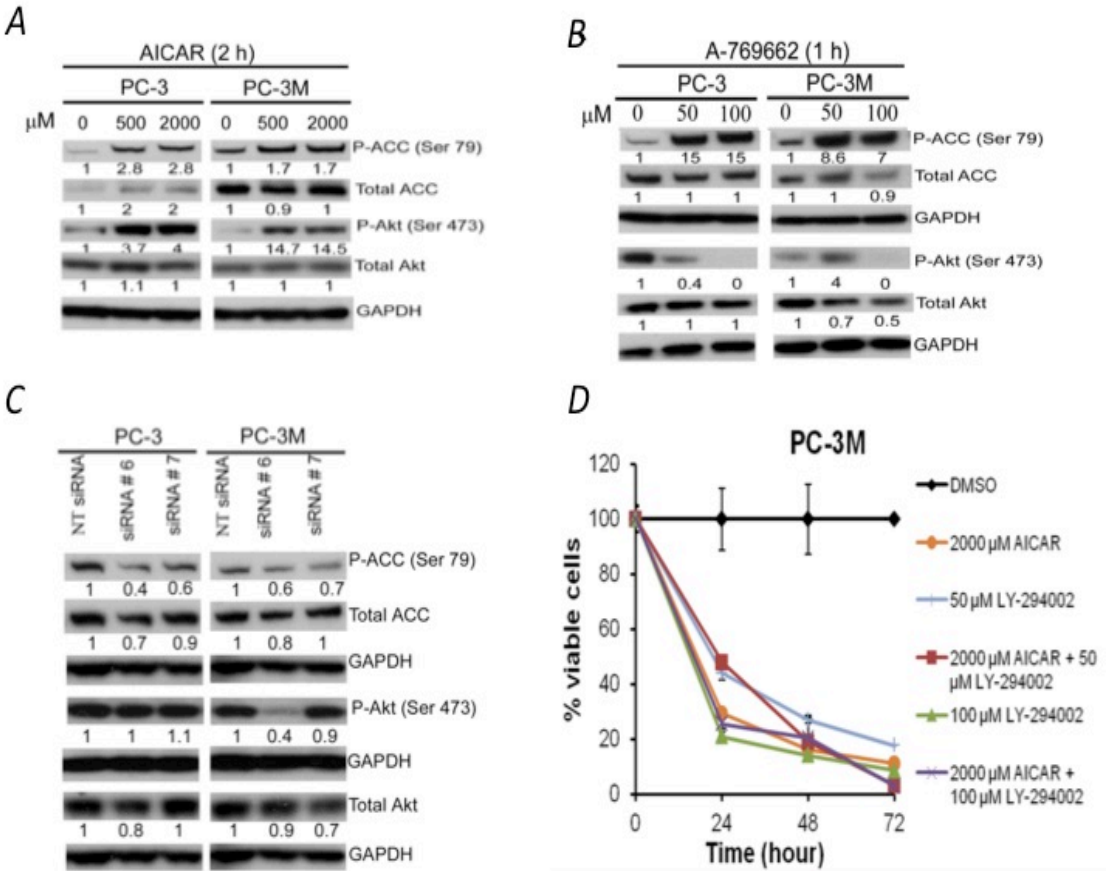
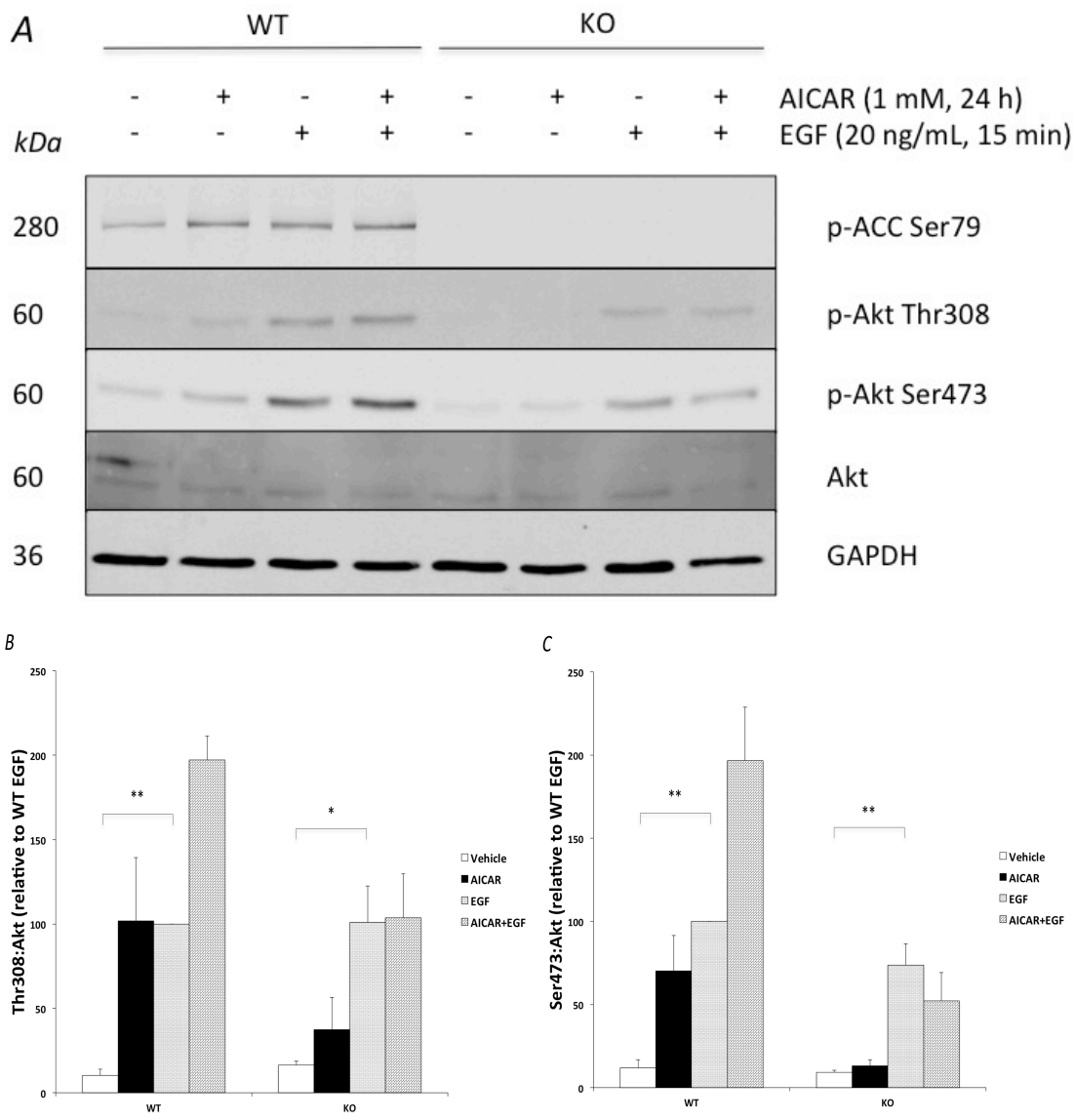


Figure 6.3 Analysis of the effect of AMPK activators on PI3K pathways in prostate cancer cells

PC3 and PC3M cells were maintained in serum-free medium before incubation with (A) AICAR (500 and 2 mM) for 2 h, (B) A769662 (50 and 100 μM) for 1 h or (C) siRNA targeting the *PRKAA1* gene. Protein lysates were made and blots were developed with the appropriate antibodies as indicated. Values under blots represent level of each protein level normalised to GAPDH. (D) PC3M cells were treated with AICAR and/or LY294002 for the indicated time period at different concentration. Viability assay was conducted using the WST-1 reagent, data represented as mean ± SD relative to the start time point. These experiments were conducted by Dr Yashmin Choudhury (University of Glasgow).

6.2.2 Analysis of the role of AMPK on EGF-stimulated Akt signalling using AMPKα1$^{-/-}$ AMPKα2$^{-/-}$ mouse embryonic fibroblasts

To assess the role of AMPK in AICAR- and A769662- mediated Akt phosphorylation, adenoviruses expressing DN mutant AMPK were studied in PC cell lines. It was however unable to demonstrate any significant down-regulation of AMPK activity. The AMPK-dependence of the inhibitory actions of AICAR and A769662 was therefore examined using WT and *AMPK α1$^{-/-}$ AMPK α2$^{-/-}$* KO mouse embryonic fibroblasts (MEFs). As expected, AMPK activity is abolished in the *AMPK α1$^{-/-}$ AMPK α2$^{-/-}$* KO MEFs as indicated by the lack of its substrate, phospho-ACC Ser79. In MEFs, EGF stimulated robust Akt phosphorylation at both Thr308 and Ser473. Unlike in PC3 cells, neither AICAR nor A769662 reduced EGF-stimulated Akt Thr308 or Ser473 phosphorylation in WT MEFs or *AMPK α1$^{-/-}$ AMPK α2$^{-/-}$* KO MEFs. Interestingly, AICAR has shown a trend in increasing Akt phosphorylation at both Thr308 and Ser473 in WT MEFs. In *AMPK α1$^{-/-}$ AMPK α2$^{-/-}$* KO MEFs, however, AICAR had no effect, suggesting this potential stimulatory effect of AICAR in MEFs may be AMPK-dependent (Figure 6.4).


A
WT
KO
AICAR (1 mM, 24 h)
EGF (20 ng/mL, 15 min)
kDa
280
60
60
60
36
p-ACC Ser79
p-Akt Thr308
p-Akt Ser473
Akt
GAPDH
B
Thr308:Akt (relative to WT EGF)
Vehicle
AICAR
EGF
AICAR+EGF
WT
KO
C
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EGF
AICAR+EGF
WT
KO

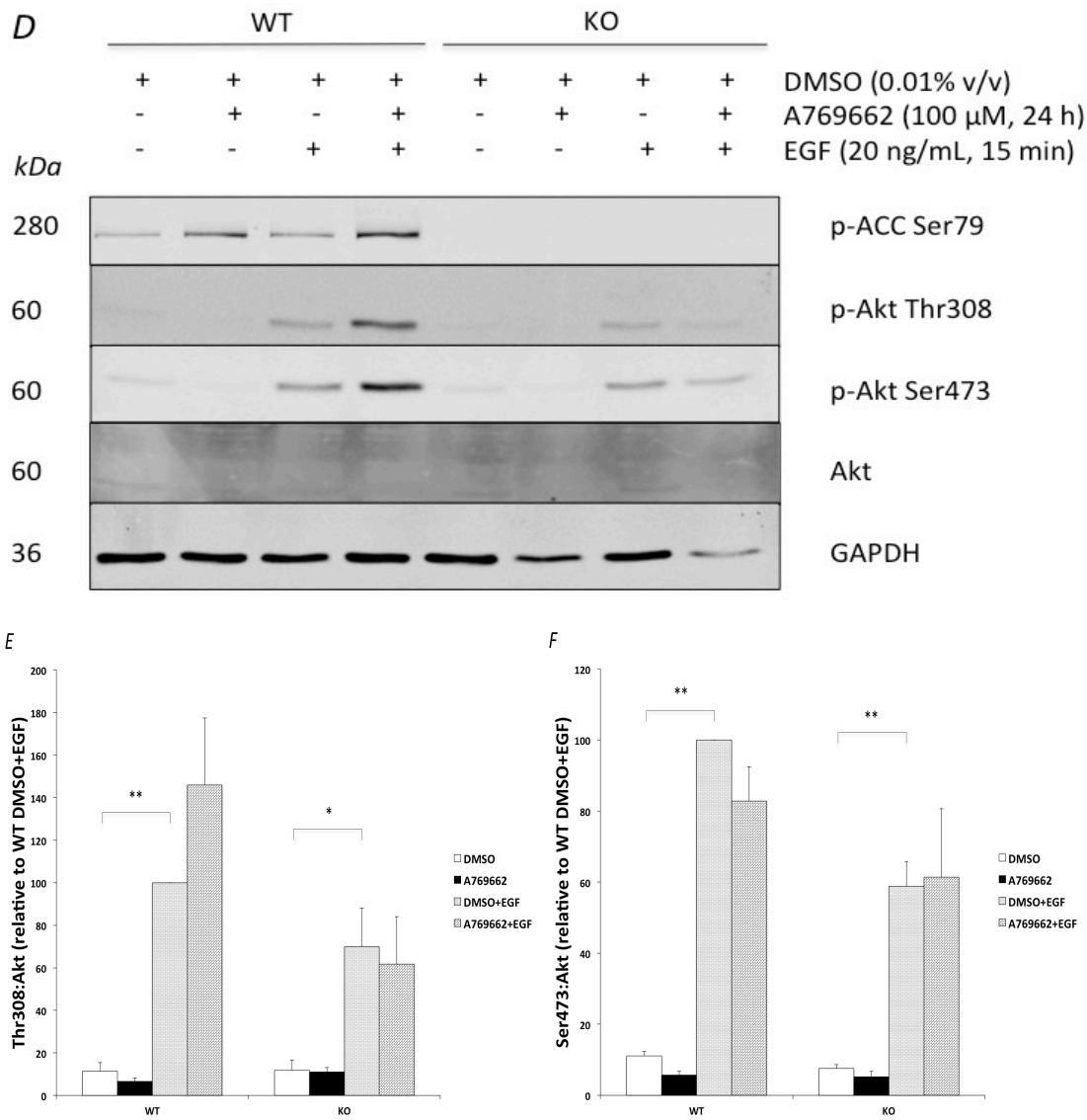

Figure 6.4 Long-term effects of AMPK activators on EGF-stimulated Akt phosphorylation in wild type (WT) and *AMPKα1*$^{-/-}$ *AMPKα2*$^{-/-}$ knock out (KO) mouse embryonic fibroblasts (MEFs)

MEFs were incubated for 2 h in serum-free medium before incubation with (A-C) 1 mM AICAR or (D-F) 100 μM A769662 for a further 24 h. EGF (20 ng/mL) was added 15min prior to lysis. Protein lysates were resolved by SDS-PAGE and subjected to immunoblotting with the antibodies indicated. GAPDH was used as loading control. (A, D) Representative blots are shown. (B, E) Densitometric analysis of phospho-Akt Thr308 level (*: $p<0.05$, **: $p<0.01$, N=3), (C, F) Densitometric analysis of phospho-Akt Ser473 level (**: $p<0.01$, N=3).

6.3 Discussion

In both early and late stage PC, gene mutations or deletions leading to activation of the PI3K/Akt pathway is the most common genetic defect (Barbieri *et al.*, 2013, Epstein and Lotan, 2015). The deregulation of the PI3K/Akt signalling pathway is associated with more advanced disease and poor prognosis (Frank and Miranti, 2013, Stephenson and Klein, 2016).

As demonstrated in Chapter 4, Figure 4.16, long-term AMPK activation decreased EGF-stimulated phospho-Akt Ser473 in PC3 cells when assessed on an apoptosis signalling array. When investigating these long-term effects of AMPK activators further by standard immunoblotting in this chapter, these findings were reinforced, with both AICAR and A769662 reducing EGF-stimulated phospho-Akt Ser473, yet EGF-stimulated phosphorylation of Akt at Thr308 was not significantly reduced. Furthermore, short-term AMPK activation experiments showed that A769662 reduced both Akt Ser473 and Thr308 phosphorylation, whereas AICAR has no effect. Activation of Akt requires phosphorylation of Ser473 in the hydrophobic motif and phosphorylation of Thr308 in the activation loop (Alessi *et al.*, 1997, Vanhaesebroeck and Alessi, 2000, Sarbassov *et al.*, 2005). In non small cell lung carcinoma tissue, it has previously been demonstrated that Ser473 phosphorylation does not always correlate with Thr308 phosphorylation (Vincent *et al.*, 2011). The study of Vincent and co-workers also proposed that phospho-Akt Thr308 is a better indicator of Akt activity than phospho-Akt Ser473 (Vincent *et al.*, 2011). It is possible that EGF-stimulated phospho-Akt Ser473 can be reduced by AMPK activation, but the effect of AMPK activation on EGF-stimulated phospho-Akt Thr308 is minimal. The two phosphorylation sites of Akt are regulated differently as previously mentioned, with Ser473 being phosphorylated by mTORC2, whereas Thr308 is phosphorylated by PDK1 (Toschi *et al.*, 2009). It has been reported that AMPK inhibits mTORC1 by phosphorylating TSC2 and raptor (Inoki *et al.*, 2003, Gwinn *et al.*, 2008). But there is no evidence so far to suggest that AMPK could influence mTORC2 in any way. Therefore, it would be interesting to see whether AMPK activators can regulate mTORC2. Also, in order to determine if Akt activity was actually altered, the

phosphorylation level of an Akt substrate, such as glycogen synthase kinase 3 (GSK3) (Cross *et al.*, 1995) could be studied.

Previously, Gan and colleagues showed that inhibition of Akt in PC3 and DU145 cells could eliminate EGF driven cell migration (Gan *et al.*, 2010). Interestingly, using PC3 and PC3M cell lines in parallel, the anti-proliferative effect of AMPK activation is independent of PI3K/Akt pathway, and inhibition of the PI3K/Akt pathway using LY294002 exerted a similar effect as incubation with AICAR alone in terms of cell viability (Choudhury *et al.*, 2014). This indicates that the effects of AMPK activators on cell viability are unlikely to be mediated by Akt inhibition, although whether AMPK activator-stimulated Akt inhibition underlies the anti-migratory effect of AMPK activators was not tested in this project.

A different approach was used to examine whether the observed effects of AICAR and A769662 are AMPK-dependent. Initially experiments with adenoviruses expressing a DN AMPK α1 were unsuccessful, such that WT and *AMPK* $\alpha 1^{-/-}$ *AMPK* $\alpha 2^{-/-}$ KO MEFs were therefore used as an alternative genetic down-regulation of AMPK. Interestingly AICAR stimulated rather than inhibited phospho-Akt Ser473 and phospho-Akt Thr308 phosphorylation in WT MEFs but not in *AMPK* $\alpha 1^{-/-}$ *AMPK* $\alpha 2^{-/-}$ KO MEFs. This may suggest that this effect in MEFs is AMPK-dependent. In WT MEFs, 24 h incubation with AMPK activators had no effect on EGF-stimulated Akt phosphorylation at either Ser473 or Thr308, such that whether the effects of AMPK activators on EGF-stimulated phospho-Akt Ser473 observed in PC3 cells are AMPK dependent or not remains elusive. This may suggest that the effect of AMPK activators (or AMPK activation) on EGF-stimulated Akt phosphorylation is a cell-type specific phenomenon. More importantly, as MEF cells are not derived from tumours, they are unlikely to have a similar molecular profile in terms of the PI3K/Akt pathway abnormalities usually seen in malignant cells. If this is true, it could be a therapeutically useful property as AMPK activators might be utilised to target Akt inhibition in malignant cells only.

Chapter 7. Final discussion

7.1 Project overview and summary of results

This project as a whole aimed to analyse the effect of AMPK activators on prostate cancer (PC) cell function, and to determine the underlying mechanism(s) of their effects. The principal findings of this study were AMPK activators could decrease PC cell proliferation and migration. And these effects are likely to be, at least partially, AMPK-dependent. The mechanism of action is possibly through the inhibition of the MAPK and PI3K/Akt signalling pathways.

The role of AMPK in terms of prostate carcinogenesis is not fully understood. Six cell lines including two AR-dependent cell lines (CWR22, LNCaP) and four AR-independent cell lines (PC3, PC3M, DU145, LNCaP-AI) were used in the project to provide an initial comparison of the basal levels and phosphorylation status of AMPK α at Thr172 and Akt at Ser473. In addition, the protein level of the upstream phospho-AMPK Thr172 kinases, LKB1 and CaMKK2, were also analysed, as were the levels of AMPK subunit isoforms in PC3, DU145 and LNCaP cells. These data showed that there were differences in the subunit isoform protein levels in the different PC cell lines.

The dynamics of AMPK activation *in vitro* were established and optimised in response to two structurally-unrelated AMPK activators that stimulate AMPK by distinct mechanisms. As demonstrated in Chapter 3, both AICAR and A769662 can activate AMPK in PC3, DU145 and LNCaP cells. These observations are in agreement with previous research, which showed that AICAR could activate AMPK in PC3 and LNCaP cells (Xiang *et al.*, 2004). This is likely through the well-established mechanism by which AICAR is phosphorylated to the AMP analogue ZMP, which subsequently allosterically activates AMPK and promotes activating Thr172 phosphorylation (Merrill *et al.*, 1997). AICAR also stimulated ACC phosphorylation, which is used as an assessor of AMPK activity, in DU145 cells, which lack LKB1, and is contrary to previous findings that AICAR is unable to activate AMPK in cells lacking LKB1, including HeLa cells and LKB1 KO MEFs (Hawley *et al.*, 2003, Shaw *et al.*, 2004, Hurley *et al.*, 2005). This finding however, supports a previous report in which AICAR activated AMPK in DU145 cells

independent of LKB1 (Yun *et al.*, 2005). The mechanism was proposed to involve other AMPK kinases unidentified at the time (Altarejos *et al.*, 2005, Yun *et al.*, 2005), although a recent study suggests that mixed-lineage kinase 3 might be the upstream kinase involved (Luo *et al.*, 2015). Indeed, allosteric effect can also play an important part as suggested by Gowans and co-workers (Gowans *et al.*, 2013). Before the start of this project, the effects of A769662 had not been examined in PC cell lines, although a recent study has reported that A769662 activates AMPK in PC3 and LNCaP cells (Zadra *et al.*, 2014), which supports the findings presented in this project.

Clinical samples were also analysed by my colleagues during this study to understand the association of AMPK activity with PC in a clinical context (Choudhury *et al.*, 2014). Interestingly, the Gleason score was positively correlated with the extent of phospho-AMPK Thr172, with a significant increase in AMPK phosphorylation in samples with a Gleason score >7 (Grade IV and V) compared to samples with a Gleason score <7 (Grade I to III). It is difficult to say whether this correlation is due to AMPK activity contributing to cancer progression or whether the increase in phosphorylation is a consequence of cancer progression. Others have reported a higher prevalence of AMPK activation (as assessed by phospho-ACC Ser79) in human PC tissue compared to normal prostate tissue, although this was not related to the Gleason score (Park *et al.*, 2009). The same group also suggested that inhibition of AMPK decreased PC cell line proliferation (Park *et al.*, 2009). However, in their study, siRNA targeting *PRKAA1* was able to reduce AMPK levels, it was unable to abolish ACC phosphorylation, which is similar to the findings presented in this project. Furthermore, Park and co-workers used compound C to inhibit AMPK and demonstrate AMPK-dependence, yet compound C has many off-target effects which inhibits a number of other protein kinases with greater efficacy than it inhibits AMPK (Viollet *et al.*, 2010). In contrast, in animal model studies, a lack of LKB1 (and therefore AMPK activity) was found to increase tumourigenesis, including PC, in mice lacking PTEN, whereas treatment of mice lacking PTEN with AMPK activators including A769662 reduced the onset of tumourigenesis in the prostate and other tissues (Huang *et al.*, 2008). Recent studies have indicated that

it is not just PC that is associated with increased AMPK activity or phosphorylation. In human glioma, AMPK activation is also associated with higher grade lesions (Liu *et al.*, 2014). In contrast, other tumours including human colon adenocarcinoma and breast cancer have been reported to exhibit an inverse relationship between phospho-AMPK Thr172 and tumour grade and clinical stage (Baba *et al.*, 2010, Migita *et al.*, 2013). Taken together, it is clear that AMPK activity varies in different cancer types and at different stages, such that there is no universal relationship between AMPK activity and tumourigenesis. The data presented in this project are the first evidence showing that AMPK activity correlates with the Gleason score in human PC and further histological studies are warranted in order to understand this observation.

As mentioned before, Park and co-workers showed compound C and transfection with siRNA targeting AMPK decreases proliferation of LNCaP and 22Rv1 cells as measured by cell count and BrdU assay *in vitro* (Park *et al.*, 2009). In the current study, using different experimental approaches including WST-1 and BrdU assays, it is evident that both AICAR and A769662 exert anti-proliferative effects in PC3 and DU145 cells and these effects might be at least partially AMPK-dependent. In addition, there was a difference in the extent of the anti-proliferative actions of AMPK activators when assessed by the WST-1 and BrdU assays, with a more marked inhibition by AMPK activators observed in the WST-1 assays. This indicates that AMPK activator-mediated inhibition of cell viability when assessed by WST-1 includes an effect in addition to the anti-proliferative actions of AMPK activators. This may reflect altered metabolism by AMPK activators leading to inhibition of NAD(P)H synthesis. It is therefore possible that the previously published results using the WST-1 assay or similar assays are in fact an observation of altered metabolism rather than dramatic change in proliferation. In other studies, AICAR has been reported to inhibit cell proliferation by inducing cell cycle arrest and apoptosis in various cancer cell types including PC *in vitro* and *in vivo* (Xiang *et al.*, 2004, Rattan *et al.*, 2005, Guan *et al.*, 2007, Sengupta *et al.*, 2007, Zhou *et al.*, 2009). These studies reported that the anti-proliferative effect produced by AICAR is likely to be AMPK-dependent and the mechanism includes inhibition of Akt, MAPK, p21, p27 and p53 signalling pathways (Xiang *et al.*, 2004,

Rattan *et al.*, 2005, Guan *et al.*, 2007, Sengupta *et al.*, 2007, Zhou *et al.*, 2009). In contrast, the anti-proliferative effect produced by AICAR has also been reported to be independent of AMPK such that it may be cancer cell type specific (Jose *et al.*, 2011). In non-cancer cells, AICAR has also been demonstrated to decrease cell proliferation in an AMPK-dependent manner, which involves cell cycle arrest and inhibition of the MAPK signalling pathway (Nagata *et al.*, 2004, Igata *et al.*, 2005). Before the start of this project, the effects of A769662 on the proliferation of PC cell lines had not been tested, although an *in vivo* study had reported that A769662 delayed tumour onset in PTEN deficient mice (Huang *et al.*, 2008). In contrast, a recent study reported that A769962 has no effect on cell proliferation in glioma cells (Liu *et al.*, 2014). In MEFs, A769662 led to a significant decrease in proliferation at high concentrations. This may well reflect a cytotoxic effect that is independent of AMPK. This effect may be cell type specific to MEFs through inhibition of proteasome as has been previously observed (Moreno *et al.*, 2008). At lower concentrations (30 μM) however, it is possible that A769662 exerted its anti-proliferative effect in an AMPK-dependent manner as shown in Chapter 4. To conclude, data in this study suggest that reduced cell proliferation in PC by AICAR is not AMPK dependent whereas similar effects induced by A769662 is likely to be AMPK-dependent.

Different approaches were used in the current study to assess cell migration, including wound healing, cell tracking and dual-chamber transwell chemotaxis assays. Prior to this work, Frigo and co-workers reported that AICAR increased migration of LNCaP cells measured by the transwell assay (Frigo *et al.*, 2011). In another study, it was also reported that AICAR increased migration of PC3 cells using a similar technique (Tang and Lu, 2009). However, the mechanism of the increased migration was not further investigated in either of those studies. AICAR has been reported to decrease both chemokinesis and chemotaxis in human monoblast-type (U973) cells (Kanellis *et al.*, 2006). In melanoma cells, AICAR also decreases cell migration in an AMPK-dependent manner (Kim *et al.*, 2012). In the current project, AICAR markedly inhibited cell chemokinesis and chemotaxis in both PC3 and DU145 cells, in agreement with the studies of Kim and co-workers in melanoma cells (Kim et al., 2012). The current study is the first to investigate

the effect of A769662 on PC cell migration. A769962 had similar inhibitory effects to AICAR in PC3 but not in DU145 cells. It was also found that AICAR is more effective in PC3 cells compared to PC3M cells in terms of anti-migration. These findings suggest that AMPK activation may not underlie the anti-migratory effect of AICAR in DU145 cells, as these were not recapitulated by the alternative AMPK activator, A769662. Taken together, cell viability, proliferation, migration, motility and chemotaxis can be suppressed by AMPK activators, particularly AICAR. The AMPK-dependence of these effects remains elusive, however. Of note, an article published shortly before the end of this project demonstrated strong *in vitro* and *in vivo* evidence that AMPK activation by a novel direct activator MT 63-78 inhibits PC growth and enhanced the growth inhibitory effect of AR inhibitor in CRPC (Zadra *et al.*, 2014).

The mechanism(s) by which AMPK activators suppress proliferation, viability and migration in PC cell lines has not been defined, yet several lines of evidence indicate that AMPK activators suppress MAPK signalling. Briefly, it has been reported that AMPK can inhibit various MAPK signalling pathways such as ERK1/2, JNK and p38 in several different non-cancerous cells (Jeong *et al.*, 2009, Qi *et al.*, 2009, Dong *et al.*, 2010, Green *et al.*, 2011b). In particular, both AICAR and A769662 have been reported to be able to decrease ERK1/2 activity *in vitro* (Green *et al.*, 2011b, Kim *et al.*, 2012). Studies also suggest that JNK might be an upstream regulator of AMPK in PC cells (Jung *et al.*, 2008). One study has suggested that AMPK is the upstream regulator of ERK5 (Young *et al.*, 2009). Data in the current study show that short-term incubation with AMPK activators has no significant effect on EGF-stimulated ERK1/2, p38 and JNK phosphorylation in PC cell lines, despite AMPK being robustly activated. In contrast, long-term stimulation of PC3 cells with AMPK activators decreased EGF-stimulated ERK1/2 phosphorylation.

Intriguingly, both AICAR and A769662 decreased EGF-stimulated ERK5 phosphorylation in PC3, DU145 and LNCaP cells. Furthermore, the phosphorylation of ERK5 by EGF was qualitatively different to that stimulated by constitutive MEK5 activity and phosphorylation of ERK5 by constitutive MEK5

was not inhibited by AMPK activators. This indicates that AMPK activation is acting at or above the level of MEK5 in the signalling pathway. Interestingly, Nagata and co-workers suggested that AMPK activation inhibits ERK1/2 at a level above MEK1/2 (Nagata *et al.*, 2004). As cross talk between ERK5 and ERK1/2 pathways has been suggested (Mody *et al.*, 2001, Barros and Marshall, 2005, McCracken *et al.*, 2008), it is also possible that AMPK activation inhibits EGF-stimulated ERK1/2 and ERK5 phosphorylation through a similar mechanism. As demonstrated by the Leung group, ERK5 is particularly important in PC in terms of promoting carcinogenesis (McCracken *et al.*, 2008, Ramsay *et al.*, 2011). The findings in this project may therefore contribute to a targeted approach in PC treatment. In addition to the above findings, it is also noticed that AICAR and A769662 both have AMPK-independent effects on MAPK signalling in PC cell lines, with AICAR decreasing ERK5 mobility and A769662 stimulating ERK1/2. In MEF cells, neither AICAR nor A769662 had any effect on EGF-stimulated MAPK phosphorylation. This may suggest that AMPK inhibits MAPK signalling by a cell type specific mechanism.

Studies of the PI3K/Akt signalling pathway in PC have suggested that this plays a vital role in cancer progression, and suppressing the pathway is considered a therapeutic target (Zhuang *et al.*, 2002, Gao *et al.*, 2003). Increased phosphorylation of Akt at Ser473 has been linked to poor clinical outcome of PC (Kreisberg *et al.*, 2004). Also, high grade PC is associated with high Akt phosphorylation (Malik *et al.*, 2002, Shukla *et al.*, 2007). Interestingly, evidence has suggested that PI3K/Akt and ERK1/2 signalling pathways might be able to compensate for each other in PC, it is also suggested dual inhibition of both pathways can be more effective in advanced PC (Malik *et al.*, 2002, Kreisberg *et al.*, 2004, Kinkade *et al.*, 2008). In addition, alterations in PTEN activity have been observed in PC cell lines including PC3 and LNCaP (Li *et al.*, 1997, Steck *et al.*, 1997). This has been studied in the context of cooperative pathway interaction between the PI3K/Akt and ERK1/2 in the Leung lab (Patel *et al.*, 2013). Indeed, inhibition of the PI3K/Akt/mTOR pathway remains an established therapeutic approach and has been investigated in both pre-clinical and clinical settings since late 20th century (Majumder and Sellers, 2005, Sarker *et al.*, 2009). During the

current study, the effect of AMPK activation on PI3K/Akt signalling was investigated, suggesting that the effect of AMPK on cell proliferation is independent of the PI3K/Akt pathway (Choudhury *et al.*, 2014). Long-term stimulation of PC cell lines with AMPK activators inhibited phospho-Akt Ser473, whereas phospho-Akt Thr308 was only inhibited by A769662. This difference between AMPK activators may suggest an AMPK-independent effect. An interesting observation is that AICAR increased phospho-Akt level at both Ser473 and Thr308 in MEFs, which is likely to be an AMPK-dependent effect as it was absent in MEFs lacking AMPK. It is possible that AICAR is unable to inhibit the PI3K/Akt signalling pathway in PC due to this effect. Previous studies also showed that AICAR can activate Akt at Ser473 and Thr308 in an AMPK-dependent manner in leukaemia cells (Leclerc *et al.*, 2010).

Taken together, the data presented in this study suggest that AMPK activation has different effects on MAPKs and PI3K/Akt in a context and activator-dependent manner, although inhibition of ERK1/2, ERK5 and Akt in the long-term may underlie the effects of AMPK activators on proliferation, viability and migration (Figure 7.1). Further studies are required to identify the mechanisms underlying these effects. Indeed, AMPK might "switch" its function in a specific metabolic and/or signalling condition, such that both anti- and pro- cancer effects can be observed even in the same tumour type. The *in vitro* experiments demonstrated an overall anti-tumourigenic effect upon AMPK activation in PC, which is possibly, at least partially through the dual inhibitory effects on both MAPK (mainly the ERK5 pathway) and PI3K/Akt signalling pathways (Figure 7.2).

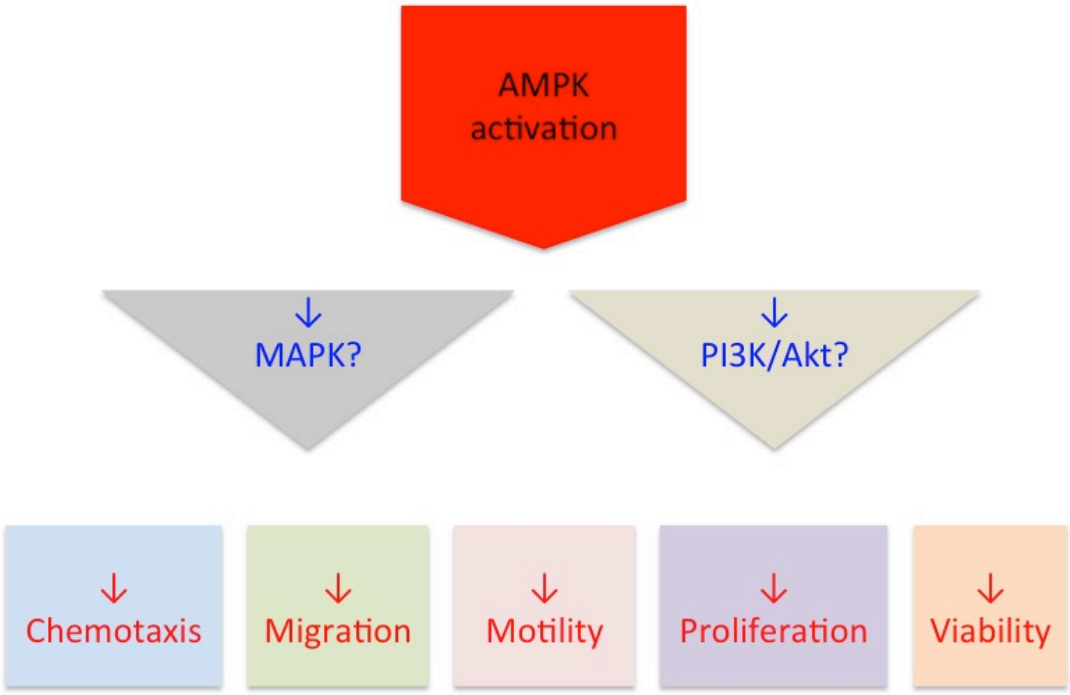

Figure 7.1 Potential effects of AMPK activation on cellular function *in vitro*
Upon AMPK activation, both MAPK and PI3K/Akt signalling pathways may be down-regulated, which in turn may lead to altered PC cell function including decreased chemotaxis, migration, motility, proliferation and viability *in vitro*.

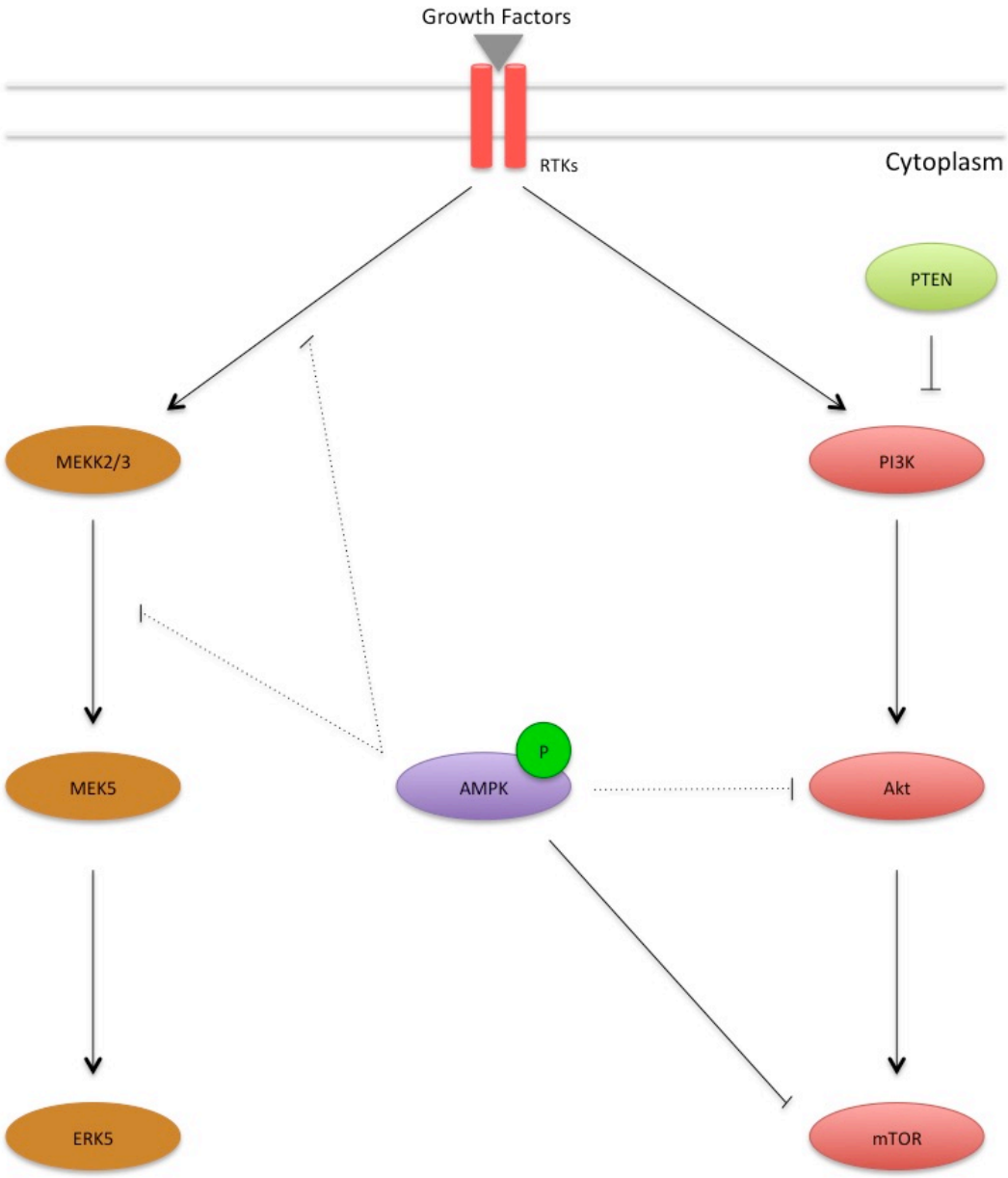

Figure 7.2 Potential mechanisms of AMPK with the ERK5 and PI3K/Akt signalling pathways

AMPK down-regulates EGF-stimulated ERK5 signalling pathway at or above the level of MEK5. It also down-regulates the PI3K/Akt signalling pathway by inhibiting EGF-stimulated Akt and mTOR. Arrow-headed lines denote activation and bar-headed lines denote inhibition, dotted lines denote potential effects. Different colours indicate different signalling pathways.

7.2 Future prospects

7.2.1 How do AMPK activators influence prostate cancer cell invasion and metastasis?

Invasion is another important cell function in PC progression, and ERK5 plays a vital part in this (Ramsay *et al.*, 2011). Whether AMPK activation inhibits cell invasion remains to be tested. Similar experiments should be carried out using Matrigel (an artificial extracellular environment), an approach that has been optimised in PC in the Leung lab (Ramsay *et al.*, 2011). Furthermore, the IncuCyte (a live cell analysis system) provides real-time high-throughput analysis of proliferation, migration, invasion and/or metastasis, which has been used in PC before (Bjorkman *et al.*, 2012, Gujral *et al.*, 2014, Harma *et al.*, 2014). The IncuCyte system can also be used in a three-dimensional approach that mimics the *in vivo* environment and the effect of AMPK activators could be examined in this manner.

7.2.2 Do AMPK activators affect mitosis/cytokinesis?

Recently, Vazquez-Martin and colleagues have reported that AMPK could be considered as a tumour suppressor in the mitotic/cytokinetic phase of the cell cycle in an energy independent manner (Vazquez-Martin *et al.*, 2009a, Vazquez-Martin *et al.*, 2009b, Vazquez-Martin *et al.*, 2011, Vazquez-Martin *et al.*, 2012, Vazquez-Martin *et al.*, 2013). Although the mechanism of this is poorly characterised, Pinter and co-workers suggest that specific AMPK subunits play an important part (Pinter *et al.*, 2012). Preliminary research in the Salt lab also suggests that AMPK activation inhibit cytokinesis in MEFs. Therefore there is a potential to explore this area using models established in this study, examining the numbers of binucleate cells (a measure of cytokinesis failure) and the localisation of AMPK in PC cell lines during the cell cycle.

7.2.3 What genetic effects do AMPK activators have and would AMPK activators have any additive effect with other current anti-cancer drugs?

The advance in research technology has created a new era in cancer research. Systematic high-throughput genotyping provides invaluable information, which can guide cancer classification and therapeutic intervention (Thomas *et al.*, 2007). Using a siRNA library approach, it is possible to identify underlying gene interactions involved in cancer progression (Bjorkman *et al.*, 2012). Similarly, experiments can be designed to look at the metabolic profile of PC cells treated with AICAR or A769662 by using the high-throughput siRNA screening. Further experiments should also be carried out to study whether activation of AMPK by AICAR or A769962 exert any synergetic or antagonistic effect with clinically approved anti-cancer drugs by using established siRNA libraries (Shanks, 2014).

7.2.4 What is the ERK5 activity in prostate cancer and how do AMPK activators alter this?

Giving the evidence that AMPK activation can decrease EGF-stimulated ERK5 phosphorylation, it remains unclear what the underlying mechanism is. In order to further address this, it is important first to determine what precisely the EGF-stimulated ERK5 activity in PC is. Analysis of ERK5 activity has proved difficult in this project as EGF and constitutive MEK5 stimulate different ERK5 species as assessed with an anti-phospho-ERK5 antibody. Further characterisation of ERK5 phosphorylation in response to EGF (and the effects of AMPK activators) may be performed using the Phos-tag SDS-PAGE system, which allows greater analysis of phosphoproteins (Kinoshita *et al.*, 2009, Kinoshita *et al.*, 2012). Nithianandarajah-Jones and co-workers have optimised this method for the separation of phospho-ERK5 species in HeLa and human endothelial cells (Nithianandarajah-Jones and Cross, 2015). Once EGF-induced ERK5 phosphorylation is analysed using this method, MEK5 inhibitors can then be used to examine whether inhibition of this pathway alone or together with AMPK activation could have similar or synergetic effects. There are currently three

inhibitors targeting the ERK5 signalling pathway, the MEK5 inhibitors BIX02188 and BIX02189 (Tatake *et al.*, 2008) and the direct ERK5 inhibitor XMD8-92 (Yang *et al.*, 2010). By using these molecular tools, the cross talk between ERK5 and other signalling pathways, including AMPK can be examined. Also, as ERK5 may activate Akt through an unknown mechanism (Datta *et al.*, 1997, Holmes *et al.*, 2007, Lennartsson *et al.*, 2010, Roberts *et al.*, 2010, Razumovskaya *et al.*, 2011), it is worthwhile to analyse these effects. Furthermore, combination therapy targeting two or more signalling pathways is found to be more effective and less toxic (Shah *et al.*, 2007, Stommel *et al.*, 2007), whether this remains the case for ERK5 and AMPK remains to be tested.

7.2.5 What are the activities of AMPK, MAPK and PI3K/Akt in clinical prostate cancer and how do these correlate to disease progression?

AMPK activation was associated with progression of PC in clinical samples (Choudhury *et al.*, 2014). In addition, studies have shown that correlation exists between MAPK and PI3K activation in clinical PC (Malik *et al.*, 2002, Kreisberg *et al.*, 2004, Kinkade *et al.*, 2008). Further studies should be carried out to analyse the status of these different signalling pathways including AMPK, AR, MAPK and PI3K at different stages of the disease.

7.3 Conclusion

This study addressed the role of AMPK in the regulation of PC cell viability, proliferation, migration and signalling. The key findings of the study were that structurally-unrelated AMPK activators that activate AMPK by different mechanisms robustly inhibited viability and migration of PC cell lines. Furthermore, activation of AMPK was associated with reduced ERK1/2, ERK5 and PI3K/Akt signalling. The AMPK-dependence of these effects, however, remains uncertain.

Appendix

Appendix 1. Manufacturers

Abcam

330 Cambridge Science Park

Cambridge

CB4 0FL

United Kingdom

Tel: +44 (0) 1223 696 000

Fax: +44 (0) 1223 215 215

Email: orders@abcam.com

Website: http://www.abcam.com

BD

Edmund Halley Road

Oxford Science Park

Oxford

OX4 4DQ

United Kingdom

Tel: +44 (0) 1865 781 666

Fax: +44 (0) 1865 781 627

Email: bduk_customerservice@europe.bd.com

Website: http://www.bd.com

Biotium

3159 Corporate Place

Hayward

CA 94545

USA

Tel: +1 510 265 1027

Fax: +1 510 265 1352

Email: order@biotium.com

Website: http://biotium.com

Cell Signaling Technology Inc.

3 Trask Lane

Danvers

MA 01923

USA

Tel: +1 978 867 2300

Fax: +1 978 867 2400

Email: info@cellsignal.com

Website: http://www.cellsignal.com

Covance

The Clove Building

4 Maguire Street

London

SE1 2NQ

United Kingdom

Tel: +44 (0) 203 810 6000

Fax: +44 (0) 207 403 7096

Website: http://www.covance.com

GeneTex

2456 Alton Parkway

Irvine

CA 92606

USA

Tel: +1 949 553 1900

Fax: +1 949 309 2888

Email: info@genetex.com

Website: http://www.genetex.com

Genova Life Science

Bibby Scientific Limited

Beacon Road, Stone

Staffordshire

ST15 0SA

United Kingdom

Tel: +44 (0) 1785 812 121

Fax: +44 (0) 1785 810 405

Email: info@bibby-scientific.com

Website: http://www.jenway.com

Lonza

8830 Biggs Ford Road

Walkersville

MD 21793

USA

Tel: +1 800 638 8174

Fax: +1 301 845 2924

Email: scientific.support@lonza.com

Website: http://www.lonza.com

Merck Millipore

Boulevard Industrial Road

Padge Road, Beeston

Nottingham

NG9 2JR

United Kingdom

Tel: +44 (0) 115 943 0840

Fax: +44 (0) 870 900 4644

Email: ukcustomerservice@merckgroup.com

Website: http://www.merckmillipore.com

Nikon

380 Richmond Road

Kingston Upon Thames

Surrey

KT2 5PR

United Kingdom

Tel: +44 (0) 208 247 1717

Fax: +44 (0) 208 541 4584

Email: discover@nikon.co.uk

Website: http://www.nikoninstruments.com

Olympus

KeyMed (Medical & Industrial Equipment) Ltd.

KeyMed House

Stock Road

Southend-on-Sea

Essex

SS2 5QH

United Kingdom

Tel: +44 (0) 170 261 6333

Website: http://www.olympus-lifescience.com

Roche Diagnostics Limited

Charles Avenue

Burgess Hill

West Sussex

RH15 9RY

United Kingdom

Tel: +44 (0) 144 425 6000

Website: http://www.roche.co.uk

Santa Cruz Biotechnology, Inc.

10410 Finnell Street

Dallas

Texas 75220

USA

Tel: +1 214 902 3900

Fax: +1 214 358 6070

Email: scbt@scbt.com

Website: http://www.roche.co.uk

Sigma-Aldrich Company Ltd.

The Old Brickyard

New Road

Gillingham

Dorset

SP8 4XT

United Kingdom

Tel: +44 (0) 1202 712 300

Fax: 44 (0) 1202 715 460

Email: ukorders@sial.com

Website: http://www.sigmaaldrich.com

Thermo Fisher Scientific

Life Technologies Ltd

Inchinnan Business Park

Paisley

PA4 9RF

United Kingdom

Tel: +44 (0) 141 814 6100

Fax: +44 (0) 141 814 6260

Email: ukorders@lifetech.com

Website: http://www.thermofisher.com

Tocris Bioscience

Tocris House

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Website: http://www.tocris.com

Appendix 2. Related publication

Choudhury, Y., Yang, Z., Ahmad, I., Nixon, C., Salt, I. P. & Leung, H. Y. (2014). AMP-activated kinase (AMPK) as a potential therapeutic target independent of PI3K/Akt signaling in prostate cancer. *Oncoscience,* 1**,** 446-456., advance online publication 4th June 2014. ISSN 2331-4737

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